THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XIII JUNE, 1932 No. 3

THE HYDROGEN-ION CONCENTRATION OF FOWL'S BLOOD IN ROUS' SARCOMA AND IN INFECTIONS (AVIAN TUBERCULOSIS AND SALMONELLOSIS).

M. J. A. DES LIGNERIS, M.D.BERNE, L.M.S.S.A.LOND.

From the South African Institute for Medical Research, Johannesburg, South Africa.

Forwarded for publication February 3rd, 1932.

WHAT is the effect of the presence of a malignant tumour in the organism on the pH of the blood ? The answers to this question, as they appear in the literature, mostly refer to human cases and are very contradictory ; moreover, these contradictions cannot simply be explained on the basis of the variety of methods used ; for various workers, using the same methods, report different results, and others, working with different methods, sometimes obtained similar results.

Reding (1927) and his associates maintain that in all cases, not only of actual cancer, but of pre-cancer and also in those of hereditary predisposition to cancer (members of families of cancer patients), the pH of the blood is always more alkaline than in non-cancer cases ; some authors, like W. Meyer (1927–28 ; 1931), in New York, have treated human cancer cases with methods based on this assumption, and which had in view the artificial acidification of the blood. Neither feeding with acid by the mouth nor its intravenous injection, neither the administration of calcium chloride nor of ammonium chloride, nor the prolonged breathing of oxygen saturated with carbon dioxide, has any appreciable effect on tumour growth, though it may momentarily diminish the alkalinity of the blood. In human cancer those cases which have retrogressed after an attack of erysipelas, for instance, associated with a relative blood acidity, do not prove that this acidity in itself damaged the tumour. If an otherwise comparatively healthy (though cancer-bearing) organism is artificially acidified, the organism attempts successfully to neutralize this abnormal condition and thus protects the tumour-cells, which are body-cells. This is totally different from the condition of acidity in a serious streptococcal infection, where the organism is reaching the point where the damaging influence of the micro-organisms nearly kills vital organs and—occasionally—lethally affects tumour-cells.

Many investigators have found the blood in actual cancer cases (not in pre-cancer nor in relatives of cancer patients) either definitely more alkaline

than in normal cases, or at least on the alkaline threshold of normal figures. To this category of workers belong: Menten (1917), Chambers (1923), Chambers and Kleinschmidt (1923), Sannie (1926), Sannie and Peyre (1926), Weiss, Sümegy and von Udvardy (1928), Schreus (1929), Pitts and Johnson (1930), Bischoff, Long and Hill (1930).

Others workers, such as Myers, Schmitz and Booher (1923), Corran and Lewis (1924), Lewis (1926), Louros (1926), Louros and Gaessler (1929), Woodard (1930), Guthmann and Wirz (1930), declare that there is on the average no appreciable difference in pH between cancer and non-cancer cases.

Another pH problem in cancer which has been studied by various workers and with widely varying results is the reaction, alkaline or acid, of actual tumour tissue and of fluids obtainable in and from tumours. A few workers (Goldfelder, 1929, Kobliha, 1930) have found the reaction of malignant cells to be alkaline, whilst several others (Woglom, 1922, 1924), Louros (1926), Harde and her co-workers (1926, 1927), Milone (1928), Louros and Gaessler (1929), using various methods, found the tumour-tissue to be definitely acid. Mendeleeff (1923) and others found not only cancerous tissue, but also embryonic and generally all rapidly growing tissues to be more acid than quiescent adult tissues. In tissue culture it has been found by various workers —including the author—that growing tissues and their pericellular fluids become more acid.

Kobliha (1930), working with Rous' sarcoma, found that, whilst the tumour-cells were alkaline, the surrounding area, *i. e.* the muscle, into which Rous' sarcoma had been grafted, was definitely acid. He also found that during the first two weeks following tumour inoculation the pH of the blood of fowls with Rous' sarcoma was unaltered; it became alkaline in the third week only, and the author explains this on the assumption that the tumour with its alkaline cells pours alkaline tissue fluid in increasing quantities into the blood circulation, and that the organism is unable to neutralize it.

But, on the other hand, Cori and Cori (1925) had shown, on examining the venous blood flowing from a fowl's wing in which a Rous' sarcoma was developing, that there was an excess of acid, as compared with the venous blood from the other, non-tumour-bearing wing; and this acidity was due to an appreciable excess of lactic acid; there was, in the venous blood from the tumour-bearing wing, 23 mgm. less of sugar and 16·2 mgm. more of lactic acid, as compared with the blood from the healthy wing.

AUTHOR'S EXPERIMENTS.

I decided to examine a large number of fowls, into which Rous' sarcoma was grafted, concerning the behaviour of the blood pH during the whole course of tumour development, and to compare this with the pH during the development of infectious diseases (tuberculosis, salmonellosis). This was done in order to establish, not only the behaviour of the blood pH in this transplantable tumour, but also to find out whether there is any appreciable difference in the effect on the pH of the blood between tumour development and that of infection with micro-organisms. It was thought that, whilst a similar behaviour in Rous' sarcoma and in infections would prove nothing, a different behaviour

in the two series might perhaps throw some additional light on the nature of Rous' sarcoma.

Sixty-six adult fowls were used for this experiment; they belonged to various races of fowls, and were mostly ordinary cross-breeds—"kaffir fowls," as they are called in this country. 50 fowls were inoculated with Rous' sarcoma, 6 with avian tuberculosis and 10 with *Bacilli gallinarum*.

The method used for the examination of the blood pH was that of Hastings and Sendroy (1924)—a colorimetric method—which can be employed at either room or body temperature (with corrections), and which requires only small amounts of blood (0·4 c.c.).

The blood was drawn from a wing vein into a syringe, and immediately transferred (without allowing the blood to come into contact with air) into a tube which contained the saline indicator solution under a layer of neutral paraffin oil. The mixture was then gently stirred up with a footed glass rod and centrifuged; it was thereafter inserted into the comparator block and compared with known controls. The indicator used was phenol-red. For further details Hastings and Sendroy's paper must be consulted.

This method was at first tested on various blood samples taken from the same fowl, in order to find out if the pH in a given untreated fowl showed constant figures. This was found to be the case. When blood was taken at 2-, 3- or 6-days' intervals the difference did not exceed 0·05, and this was considered a sufficient degree of constancy to allow this method to be used for further experiments. The pH was then tested for each fowl, prior to the inoculation with Rous' sarcoma and afterwards, in some cases 12–24 hours after inoculation and then at various intervals until death, which occurred between the 12th and the 23rd day after tumour inoculation.

The normal pH of fowls was found to vary from 7·42 to 7·60. The average was 7·525; over 80 per cent. of the fowls had a pH of 7·48 to 7·56. There were distinct differences between various races of fowls; it was found that Rhode Island Reds had a slightly higher pH than Leghorns (7·56 on the average against 7·50 in one series of experiments).

1. *Fowls with Rous' Sarcoma.*

The inoculation of Rous' sarcoma was done by injection of 2 c.c. of tumour pulp from a tumour-bearing fowl into the muscles of the left breast of the new bird. 12–24 hours, or 2 days later, the pH was found to be either the same as before the inoculation or (more often) very slightly more acid. It may be that in some cases the lowering of the pH was the consequence of some loss of blood along the needle canal after the withdrawal of the needle, when blood was taken. Fowl's blood clots rather slowly, and there was occasionally a loss of a few c.c. of blood. It is known from human cases that loss of blood causes a lowering of pH, which is, however, soon neutralized, and sometimes followed by an increase in alkalinity. The first indication of an increased alkalinity in the blood was usually noticed after 3–4 days, *i.e.* about one day before the developing tumour had reached palpable size; in cases where the tumour formed slowly, the increase of alkalinity also began later. These results differ from those of Kobliha (1930), who found no alteration in the blood pH

TABLE I.—*pH of Blood in*

Before inoculation.	12–24 hours after inoculation.	2 days after.	3 days.	4 days.	5 days.	6 days.	7 days.	8 days.	9 days.	10 days.	11 days.	12 days.	13 days.	14 days.
7·43	7·44	..	..	7·46	7·45	7·48	..	..	..	7·55	..	..	..	7·58
7·49	7·45	..	7·48	7·48	..	7·47	..	7·54	..	7·56	..	7·60	..	7·59
7·54	7·55	..	..	7·57	..	..	7·59	..	..	..	..	7·70	..	7·72
7·54	7·50	..	7·55	..	7·60	..	..	..	..	7·62	..	..	..	7·65
7·58	..	7·58	..	7·57	..	7·60	..	7·60	..	..	7·58	..	7·65	..
7·43	..	..	..	7·50	..	..	7·55	..	..	7·56	..	..	7·60	7·30
7·46	..	7·45	..	7·43	7·44	..	7·50	..	..	..	7·58	..	7·65	7·65
7·48	7·50	7·55	..	7·60	..	7·65	..	7·64	..	7·68	..	7·68	7·70	7·53
7·58	..	..	7·58	..	..	7·65	..	7·63	..	..	..	7·70	..	7·68
7·51	7·47	..	..	7·54	..	..	7·58	..	..	..	7·57	..	..	7·62
7·53	7·54	..	7·52	..	7·58	..	7·65	..	..	7·63	..	..	7·62	..
7·57	7·55	..	7·60	..	7·57	..	..	7·62	..	..	7·70	..	..	7·67
7·48	..	..	..	7·47	..	..	..	7·57	..	..	7·60	..	..	7·62
7·50	7·50	..	..	7·52	..	7·54	7·56	..	7·54	..	..	7·63	..	7·64
7·54	..	7·54	..	..	7·56	..	..	..	7·67	..	7·70	..	..	7·75
7·55	7·54	..	..	7·48	..	7·58	..	7·58	..	..	7·65	7·58	..	..
7·58	7·56	..	..	7·60	..	7·60	..	7·60	..	7·62	..	7·60	..	..
7·55	7·50	..	..	7·62	..	..	7·63	..	..	7·68	..	..	..	7·62
7·58	..	7·58	..	7·53	..	7·58	..	..	7·58	..	..	7·63	..	..
7·45	..	..	..	7·49	7·54	..	7·55	..	..	7·63	..	..	7·65	..
7·52	7·53	..	..	7·52	..	7·58	..	7·62	..	..	7·70	..	..	7·72
7·54	7·54	..	..	..	7·50	..	7·52	..	7·58	..	7·62	..	..	7·67
7·48	7·45	..	..	7·55	..	7·58	..	..	7·62	7·60	7·65	7·63	7·72	7·75
7·52	..	..	..	..	7·58	7·60	..	7·59	..	7·63	..	7·64	..	7·68
7·53	..	..	7·50	7·54	..	7·56	..	7·62	..	..	7·62	..	..	7·67
7·56	7·55	..	7·56	..	7·58	..	7·58	..	7·65	..	7·64	..	..	7·60
7·52	7·50	..	..	7·60	..	..	7·62	7·60	..	..	7·68	..	7·68	..
7·45	7·46	7·44	..	7·50	..	..	7·48	..	7·58	..	..	7·56	..	7·56
7·42	..	..	..	..	7·48	..	7·50	..	7·60	..	7·65	..	7·63	..
7·55	..	..	7·54	..	7·62	..	..	7·62	..	..	7·64	..	..	7·67
7·58	7·58	..	7·62	7·60	7·64	..	7·70	..	7·65	..	7·68	..	7·72	..
7·50	7·52	..	7·48	7·50	7·52	..	7·55	..	7·60	..	..	..	7·62	..
7·54	..	7·50	..	7·54	..	..	7·58	..	7·62	..	7·60	..	7·60	..

Fowls with Rous' Sarcoma.

15 days.	16 days.	17 days.	18 days.	19 days.	20 days.	21 days.	22 days.	Metastases.	Date of death (after inoculation) and Remarks.
7·49	..	..	..	..	..	..	..	None	15th day. Last pH taken 2 hours before death.
..	..	7·65	..	..	7·64	7·57	7·38	In lungs	23rd day. Last pH taken 3 hours before death.
..	..	7·70	..	7·58	..	..	..	None	20th day. Last pH taken 16 hours before death.
..	7·66	7·50	..	..	..	..	..	A few	18th day. Last pH taken 6 hours before death.
..	7·70	..	..	..	..	..	..	None	17th day. Found dead.
7·0	..	..	..	..	..	..	..	Numerous in lungs	15th day. Last pH taken immediately before death.
..	..	7·68	..	..	..	..	..	None	18th day. Found dead.
..	..	..	..	..	..	..	..	,,	14th day. Last pH taken a few hours before death.
..	..	7·67	..	..	7·38	7·12	..	In lungs	21st day. Last pH taken 3 hours before death.
..	7·57	7·62	..	..	..	..	..	Some in lungs	18th day. Found dead.
..	7·65	..	..	7·67	7·49	..	..	In lungs	21st day. Last pH taken 4 hours before death.
7·65	..	..	..	..	..	..	..	None	16th day. Found dead.
7·58	..	..	..	..	..	..	..	,,	16th day. Last pH taken 7 hours before death.
..	7·62	..	..	7·67	..	7·68	7·67	A few in lungs	23rd day. Found dead.
..	7·68	..	..	..	..	..	..	Ditto	16th day.
..	..	..	..	..	..	..	..	None	12th day. Last pH taken 4 hours before death.
7·68	..	..	..	..	..	..	..	,,	16th day. Found dead.
..	..	..	..	..	..	..	..	,,	15th day. Found dead.
7·70	..	..	7·70	7·65	..	..	..	A few	19th day. Last pH taken 3 hours before death.
..	..	..	..	..	..	..	..	None	14th day. Found dead.
..	7·70	..	7·67	..	7·68	7·32	7·20	In lungs	22nd day. Last pH taken 2 hours before death.
..	7·65	..	7·68	..	..	..	..	A few	19th day. Found dead.
..	..	..	..	..	..	..	..	None	15th day. Found dead.
..	7·70	..	7·70	7·72	7·67	..	..	Some in lungs	21st day. Found dead.
..	7·65	..	..	..	..	..	..	None	17th day. Found dead.
..	7·70	..	7·70	7·42	..	..	..	A few	19th day. Last pH taken 3 hours before death.
7·72	..	..	..	..	..	..	..	None	16th day. Found dead.
..	7·58	7·55	..	..	..	..	..	,,	18th day. Last pH taken 18 hours before death.
7·68	..	..	..	..	..	..	..	,,	16th day. Found dead.
..	7·75	..	7·70	..	7·72	7·37	7·18	In lungs	22nd day. Last pH taken 2 hours before death.
7·75	..	7·73	7·58	..	..	..	..	None	19th day. Found dead.
..	7·67	7·67	..	..	..	..	..	,,	18th day. Found dead.
7·63	..	7·62	..	..	..	..	..	,,	18th day. Found dead.

TABLE I.—*pH of Blood in Fowls*

Before inoculation.	12–24 hours after inoculation.	2 days after.	3 days.	4 days.	5 days.	6 days.	7 days.	8 days.	9 days.	10 days.	11 days.	12 days.	13 days.	14 days.
7·58	7·52	..	7·57	..	7·60	..	..	7·63	..	..	7·64	..	..	7·63
7·60	7·55	..	..	7·58	..	7·57	..	7·58	..	7·58	..	..	7·70	7·70
7·48	7·50	..	7·43	..	7·48	7·50	7·55	..	..	7·60	..	..	7·58	..
7·53	..	..	7·54	..	..	7·58	7·57	..	..	7·62	..	..	7·62	..
7·55	..	7·53	..	7·57	..	7·58	..	7·62	..	7·63	..	..	7·62	..
7·55	7·53	..	..	7·56	..	7·58	..	..	7·65	..	7·67	..	..	7·63
7·50	7·47	7·50	7·52	7·54	7·57	7·60	7·65	..	7·70	..	7·75	..	7·73	..
7·52	7·52	..	7·53	..	7·57	..	..	7·60	..	7·65	..	7·57	..	7·63
7·57	..	..	7·58	7·56	..	7·58	..	7·56	..	7·65	..	7·60	..	7·62
7·54	7·50	..	..	..	7·52	..	7·60	..	..	7·62	..	..	..	7·62
7·53	7·47	..	..	7·50	..	..	7·52	..	7·55	..	7·62	..	7·60	..
7·58	7·53	..	..	..	7·60	..	..	7·58	..	7·65	..	7·64	..	7·62
7·48	..	7·48	..	7·54	..	7·62	..	7·60	..	7·62	..	7·61	..	7·63
7·57	7·56	..	..	7·54	..	..	..	7·58	..	7·70	..	7·65	..	7·68
7·51	7·52	..	7·53	..	..	7·58	..	..	7·65	..	..	7·68	..	7·70
7·56	7·54	..	..	7·60	..	..	7·65	..	..	7·70	..	..	7·70	..
7·48	7·49	..	7·47	..	7·55	..	..	..	7·62	..	..	7·68	..	..

TABLE II.—*Average of pH in*

	Before inoculation.	12–24 hours after inoculation.	2 days after.	3 days.	4 days.	5 days	6 days.	7 days.	8 days.
pH's of fowl dying on 12th day . .	7·55	7·54	..	..	7·48	..	7·58	..	7·58
Average pH's of fowls dying on 14th day	7·465	7·50	7·55	..	7·545	7·54	7·65	7·55	7·64
Ditto on 15th day	7·495	7·482	7·50	7·52	7·529	7·513	7·560	7·607	7·57
,, 16th ,, ...	7·517	7·533	7·51	7·565	7·580	7·537	7·60	7·560	7·605
,, 17th ,, ...	7·566	7·528	..	7·550	7·570	7·60	7·57	7·65	7·594
,, 18th ,, ...	7·492	7·483	7·463	7·480	7·502	7·48	..	7·538	..
,, 19th ,, ...	7·545	7·54	..	7·525	7·565	7·54	7·54	7·59	..
,, 20th ,, ...	7·52	7·512	..	7·495	7·535	7·565	..	7·587	..
,, 21st ,, ...	7·565	..	7·53	7·58	7·57	..	7·615	..	7·625
,, 22nd ,, ...	7·53	7·515	..	7·522	7·513	7·595	7·543	7·57	7·592
Average of all 50 fowls. . . .	7·524	7·513	7·515	7·533	7·537	7·55	7·575	7·576	7·599

with Rous' Sarcoma (continued).

15 days.	16 days.	17 days.	18 days.	19 days.	20 days.	21 days.	22 days.	Metastases.	Date of death (after inoculation) and Remarks.
..	7·58	..	..	..	..	..	..	A few	17th day. Last pH taken 15 hours before death.
..	7·65	..	..	..	..	..	..	,,	17th day. Last pH taken 18 hours before death.
7·58	..	7·60	..	7·30	..	..	..	None	19th day. Last pH taken 2 hours before death.
..	7·68	..	7·65	..	7·63	7·60	..	In lungs	22nd day. Found dead.
..	7·59	..	7·70	..	7·70	..	..	A few in lungs	21st day. Last pH taken 18 hours before death.
7·59	..	..	..	..	..	..	..	None	16th day. Last pH taken 6 hours before death.
7·68	..	..	..	..	..	..	..	,,	16th day. Last pH taken 6 hours before death.
..	7·65	..	7·62	..	7·60	7·53	..	Some in lungs	22nd day.
..	7·68	..	..	..	..	..	..	None	17th day. Found dead.
7·40	..	..	..	..	..	..	..	,,	15th day. Last pH taken half an hour before death.
7·70	..	7·68	..	7·68	7·45	..	..	In lungs	20th day. Last pH taken 4 hours before death.
..	7·70	..	..	..	..	..	..	None	17th day.
7·54	..	..	..	..	..	..	..	,,	16th day. Found dead.
..	7·65	..	7·60	..	7·68	..	7·45	In lungs	22nd day. Last pH taken 1 hour before death.
7·65	..	..	..	..	..	..	..	None	16th day. Found dead.
..	7·68	..	..	..	..	..	..	,,	17th day. Found dead.
7·65	..	7·66	..	7·63	7·32	..	..	Some in lungs	20th day. Last pH taken 3 hours before death.

Fowls with Rous' Sarcoma.

9 days.	10 days.	11 days.	12 days.	13 days.	14 days.	15 days.	16 days.	17 days.	18 days.	19 days.	20 days.	21 days.	22 days.
..	..	7·65	7·58	..	..	..	..	..	..	..	..	..	..
..	7·655	..	7·68	7·675	7·53	..	..	..	..	..	..	..	..
7·657	7·602	7·667	7·63	7·683	7·589	7·457	..	..	..	..	..	..	..
7·640	7·62	7·683	7·63	7·655	7·688	7·653	7·68	..	..	..	..	..	..
..	7·64	7·63	7·62	7·70	7·643	..	7·657	7·50	..	..	..	..	..
7·60	..	7·583	7·56	7·61	7·61	7·63	7·607	7·628	..	..	..	..	..
7·603	..	7·65	..	7·65	..	7·677	7·65	7·665	7·653	7·475	..	..	..
7·685	7·63	7·62	7·69	7·61	7·72	7·675	7·65	7·68	..	7·64	7·42	..	..
..	7·63	..	7·70	7·62	7·68	..	7·59	7·67	7·70	..	7·54	7·12	..
..	7·632	7·67	7·607	7·62	7·658	..	7·686	7·65	7·648	..	7·658	7·478	7·302
7·616	7·626	7·645	7·632	7·652	7·638	7·593	7·658	7·641	7·664	7·591	7·58	7·456	7·376

for the first 15 days following tumour inoculation. In ordinary cases of Rous' sarcoma, *i. e.* when the local tumour increased to a large size, becoming to a variable extent necrotic and softened in the centre, whilst there were either no macroscopically visible metastases or only a few of them in the lungs, liver, etc., the increase of pH to 7·6–7·7 or even more continued until a short time (12 or less hours) before death. Then, when the fowl was *in extremis*, a sudden decrease of pH occurred, the pH dropping to 7·3–7·2 or lower. In those cases, where large metastases were found in the lungs, indicating a serious interference with the respiration, the pH usually began to drop some days before death; this occurred more slowly and reached rather lower figures than in those cases where metastases were few or absent. On the whole, the longer the fowls lived after tumour inoculation, the more metastases they had.

For details see Tables I and II.

2. *Fowls Inoculated with Avian Tuberculosis.*

Of 6 fowls injected intramuscularly with 1 c.c. of an emulsion of avian tuberculosis culture, 5 developed tuberculosis and died of it, whilst 1 remained healthy. It will be seen from Table III that avian tuberculosis had distinctly the effect of raising the pH, as did Rous' sarcoma, though not quite as high. In most cases, however, the pH already dropped distinctly during the month preceding death and reached low figures one or two days before death, such as were reached, in the case of fowls with Rous' sarcoma, only a few hours before death. The birds were, during that period of diminishing pH, obviously ill. From two of the birds tubercle bacilli were recovered from lesions in the liver. In most cases tubercles were very numerous.

3. *Fowls Inoculated with* Bacillus gallinarum.

Ten fowls were injected intravenously with suspensions of agar-cultures of *B. gallinarum* in saline (1 c.c. each); of these fowls, 6 died and 4 recovered, which indicates a not very virulent condition of the strain. Here, again, we noticed the initial slight diminution of pH, followed by an increase as in Rous' sarcoma and tuberculosis. In the terminal stages the pH again dropped rather acutely, and thus resembled the behaviour in Rous' sarcoma more than the behaviour in avian tuberculosis. For details see Table IV.

DISCUSSION.

Whilst it may appear somewhat risky to apply conclusions drawn from experiments with Rous' sarcoma to human cancer cases, one may be permitted to state that the results of these experiments certainly do not support the opinion advanced by Reding and his school, that an increased alkalinity of the blood is a valuable diagnostic sign in cancer; in our fowls the introduction of any living and multiplying agent, whether known micro-organisms or the unknown Rous' "virus," was followed, after a period of latency or of a slight decrease in pH value, by a distinct increase in alkalinity, and this increase continued, until the general health of the animal broke down to such an extent that the organism could not neutralize the acid products which originated

TABLE III.—*pH of Blood in Fowls with Avian Tuberculosis.*

Before inoculation.	8 days after inoculation.	15 days after.	End of first month.	6 weeks.	End of second month.	2½ months.	3 months.	3½ months.	4 months.	4½ months.	Date of death (after inoculation).
7·45	7·48	7·48	7·50	7·52	7·54	7·52	7·54	7·45	7·40	7·10	4½ months. 1 day after last pH was taken.
7·52	7·50	7·48	7·52	7·56	7·55	7·58	7·45	7·25	..	..	3½ months. Last pH taken 2 days before death.
7·51	7·55	7·52	7·54	7·56	7·58	7·60	7·60	7·52	7·40	7·05	4½ months. Last pH taken 18 hours before death.
7·49	7·50	7·55	7·56	7·60	7·58	7·56	7·42	7·18	..	..	3½ months. Last pH taken 2 days before death.
7·53	7·50	7·52	7·54	7·58	7·62	7·64	7·62	7·54	7·30	..	4 months. Last pH taken a few days before death.
7·55	7·55	7·52	7·56	7·56	7·54	7·50	7·52	7·55	7·52	..	Remains healthy. Killed 7 months after inoculation.
7·51	7·51	7·51	7·54	7·56	7·57	7·57	7·53	7·42	7·41	7·08	Average of 6 fowls.

TABLE IV.—*pH of Blood in Fowls with* B. gallinarum.

Before inoculation.	18 hours after inoculation.	48 hours after.	3 days.	4 days.	5 days.	6 days.	7 days.	Date of death (after inoculation).
7·49	7·40	7·50	7·58	7·62	7·58	..	..	6th day.
7·55	7·46	7·48	7·48	7·55	7·30	..	..	6th day. 4 hours after last pH was taken.
7·52	7·42	7·50	7·58	7·36	..	..	..	5th day. 2 hours after last pH was taken.
7·54	7·50	7·56	7·62	7·64	7·63	7·48	..	7th day. Last pH taken 6 hours before death.
7·52	7·52	7·58	7·57	7·60	7·60	7·56	7·54	Recovered.
7·50	7·44	7·48	7·50	7·52	7·50	7·52	7·50	,,
7·55	7·52	7·58	7·64	7·64	7·65	7·67	7·25	Beginning of 8th day. Last pH taken shortly before death.
7·58	7·60	7·65	7·60	7·62	7·63	7·70	7·60	Recovered.
7·44	7·40	7·48	7·52	7·55	7·50	7·10	..	7th day. Last pH taken 10 hours before death.
7·57	7·58	7·62	7·64	7·64	7·20	..	..	6th day. Last pH taken 5 hours before death.
7·53	7·48	7·54	7·57	7·57	7·51	7·50	7·47	Average of 10 fowls.

either from disintegrating tumour-cells or from micro-organisms. In more acute conditions this breakdown is more sudden; in more chronic diseases, like tuberculosis, the terminal stage is more prolonged and the final diminution of pH value reaches lower levels.

Each organism has a definite pH value, with a very slight margin of variation, which is normal and which it tries to maintain. When, as a consequence of the introduction of foreign and more acid biological products, these products have to be neutralized by the alkaline reserves of the organism, we witness the usual type of reaction of living matter, *i. e.* not only neutralization and compensation, but hyperneutralization and hypercompensation, and it is this which accounts for the increased alkalinity both in the Rous' sarcoma and the infection cases.

These experiments on the blood pH throw no light on the nature of the agent in Rous' sarcoma. It would seem unjustifiable to say that they can be interpreted as favouring the external-virus theory of cancer or even of Rous' sarcoma. Of course, both in infections with micro-organisms and in tumour inoculation, foreign living material is introduced into a new host, and this calls for complex interactions and reactions; one of the consequences of these reactions is apparently the increased alkalinity of the blood; more cannot be said. The pH behaviour does not give us any indication, whether the primary origin of the Rous' agent is to be sought in the tumour-cell or whether it came from the outside world.

SUMMARY.

1. In fowls infected with Rous' sarcoma the pH of the blood rises, and remains high until just before death.

2. The pH of the blood also rose in fowls infected with avian tuberculosis and *Bacillus gallinarum*, and fell again in the later stages of the disease.

REFERENCES.

Bischoff F., Long, M. L., and Hill, E.—(1930) *J. Pharmac. Exp. Ther.*, **39**, 425.
Chambers, W. H.—(1923) *J. Biol. Chem.*, **55**, 229.
Idem and Kleinschmidt, R. E.—(1923) *Ibid.*, **55**, 257.
Cori, C. F., and Cori, G. T.—(1925) *Ibid.*, **65**, 397.
Corran, J. W., and Lewis, W. C. M.—(1924) *Biochem. J.*, **18**, 1358.
Errera, J., Reding, R., and Slosse, A.—(1930) *C.R. Soc. Biol. Paris*, **103**, 24.
Goldfeder, A.—(1929) *Z. Krebsforsch.*, **29**, 134.
Guthmann, H., and Wirz, P.—(1930) *Arch. Gynaek.*, **142**, 530.
Harde, E., and Danysz-Michel, M.—(1926) *C.R. Soc. Biol. Paris*, **95**, 1489.
Harde, E., and Henri, P.—(1927) *Ibid.*, **96**, 535.
Hastings, A. B., and Sendroy (Jun.), J.—(1924) *J. Biol. Chem.*, **61**, 695.
Kobliha, F.—(1930) *Z. Krebsforsch.*, **32**, 544.
Lewis, W. C. M.—(1926) *Brit. Med. J.*, **2**, 920.
Louros, N. C.—(1926) *Münch. med. Wschr.*, **73**, 53.
Idem and Gaessler.—(1929) *Z. Krebsforsch.*, **28**, 191.
Mendéléeff, P.—(1923) *C.R. Soc. Biol. Paris*, **88**, 146.—(1923) *Ibid.*, **88**, 293.
Menten, M. L.—(1917) *J. Cancer Res.*, **2**, 179.

MEYER, W. (1927–28) quoted from Meyer, 1931.—(1931) *Ann. Surg.*, **93**, 25.—(1931) 'Cancer: Its Origin, its Development and its Self-perpetuation.' New York (Paul B. Hoeber, Inc.).
MILLET, H.—(1928) *J. Biol. Chem.*, **78**, 281.—(1929) *Ibid.*, **82**, 263.
MILONE, S.—(1928) *Arch. Sci. med.*, **52**, 23.
MYERS, V. C., SCHMITZ, H. W., AND BOOHER, L. E.—(1923) *J. Biol. Chem.*, **57**, 209.
PITTS, H. C., AND JOHNSON, H. R.—(1930) *New Engl. J. Med.*, **202**, 415.
REDING, R.—(1927) 'L'équilibre acide-base et l'équilibre ionique dans le cancer et le pré-cancer. Contribution à l'étude des mécanismes biologiques régulateurs de la division cellulaire.' Liège.
SANNIE, C.—(1926) *Bull. Ass. franç. Cancer*, **15**, 187.
Idem AND PEYRE, E.—(1926) *Ibid.*, **15**, 199.
SCHREUS, H. T.—(1929) *Klin. Wschr.*, **8**, 1764.
WEISS, S., SUEMEGI, S., AND VON UDVARDY, L.—(1928) *Ibid.*, **7**, 1178.
WOGLOM, W. M. H.—(1922) *J. Cancer Res.*, **7**.—(1924) *Ibid.*, **8** (quoted from Kobliha).
WOODARD, H. Q.—(1930) *J. Cancer Res.*, **14**, 319.

CORTICAL PROLIFERATION IN THE MOUSE SUPRARENAL AFTER PEPTONE.

RAYMOND WHITEHEAD.

From the Department of Pathology, Victoria University, Manchester.

Received for publication April 4th, 1932.

DUNN (unpublished observations) found that the administration of peptone to rabbits was sometimes followed by the appearance of numerous mitoses in the suprarenal cortex. Since so little is known of the functions of the suprarenal, it seemed desirable to study further the conditions under which this phenomenon occurs. No record of similar previous work with peptone could be found.

METHODS.

Mice of various colours were kept in the laboratory in metal bins or wooden boxes each holding 10 mice comfortably. Oats and water were allowed freely.

The peptone solution was injected intraperitoneally with a record syringe, the abdomen being swabbed at the site of injection before and after the operation with methylated spirit. The peptone solution and syringe were sterilized by boiling before use, the solution being allowed to cool to about the body temperature of the mouse before injection.

The surviving mice were killed by fracture of the neck. The autopsy was performed immediately, both suprarenals being first removed, and then the other organs examined macroscopically.

The routine fixative was: Potassium bichromate, 2·5 gm.; mercuric chloride, 5·0 gm.; distilled water, to 100 c.c.

This fixative is not recommended for future work, as after it the nuclei of the cortical cells stain poorly with hæmalum and the chrome reaction of the medulla is faint.

After fixation and washing in tap-water the suprarenals were dehydrated, cleared, and embedded in paraffin. Sections were cut at 4μ, and a ribbon containing the central sections mounted. As a rule the gland was cut longitudinally. The sections were stained with hæmalum and eosin.

Each was examined first with a low power, and then a central section was systematically swept for mitoses with a 1/12 oil immersion. The situation and phase of each mitosis were noted. The two members of each dispireme were reckoned as one mitotic figure.

PRELIMINARY EXPERIMENTS.

Preliminary experiments suggested that mitoses were abnormally frequent between 50 and 80 hours after dosing. On the other hand, they were rare during the first 24 hours after dosing. Thus in one experiment 80 out of 100 mice died during the first 6 hours after a heavy dose of peptone. Of these 80, 54 did not show a single mitosis, and only 2 showed more than 3 each, viz. 1 showed 4 and 1 showed 5. These results are also accepted provisionally as indicating that mitoses are rare in the suprarenals of adult mice. A systematic study of the frequency and distribution of mitoses in the normal mouse suprarenal is in progress.

CORTICAL PROLIFERATION.

Experiment 1.

Eighty-six female mice exactly 6 months old received intraperitoneal injections of a solution containing 25 gm. of Witte's peptone and 0·85 gm. of sodium chloride in 100 c.c. Each mouse received a nominal dose of 7·5 mgm. of peptone per gm. body-weight. By using a solution of the strength stated the volume injected was kept small. For example, a 17-gm. mouse would receive 0·51 c.c., a 23-gm. mouse 0·69 c.c.

After the dose here used roughly 50 per cent. of the mice were expected to die in a few hours without recovering from the general depression that peptone induces. Actually 40 of the total 86 mice were dead in 8 hours after dosing.

The suprarenals from 24 of these mice were fixed for special purposes by methods preventing accurate observation of mitoses. The numbers of mitoses found in the glands from the remaining 62 mice are shown in Fig. 1.

The noteworthy points are the large number of mice dead in 8 hours that showed no mitoses, the hint of proliferation in 1 mouse killed at 55 hours, and the unequivocal evidence of its occurrence between 71 and 97 hours, when one-half (7) of the mice showed over 10 mitoses, 1 showing 55, while only 2 showed less than 5, and none showed no mitoses. At 97 hours proliferation was less active. Its later course was not studied.

The mitoses were distributed as follows: Subcapsular layer, 113; rest of cortex, 125.

Those in the cortex below the subcapsular layer were most frequent in its outer part, rapidly decreasing in number as its inner border was approached. One mitosis occurred in a cortical stroma cell. Six mitoses were seen in the medulla. These were confined to its two outermost cell layers, and all but 1 occurred when cortical proliferation was at its acme. This observation is of interest as suggesting that peptone stimulates cell division not merely in the cortex, but also in the medulla, although to a much slighter degree.

The numbers of cortical mitoses classified according to phase were as follows: Monospiremes, 39; monasters, 25; diasters, 4; dispiremes, 170.

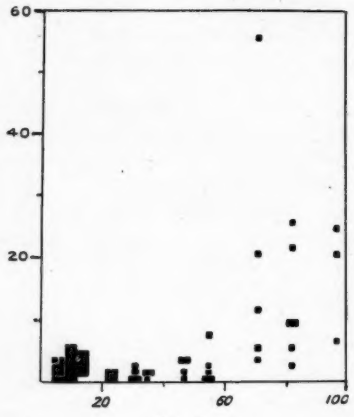


FIG. 1.

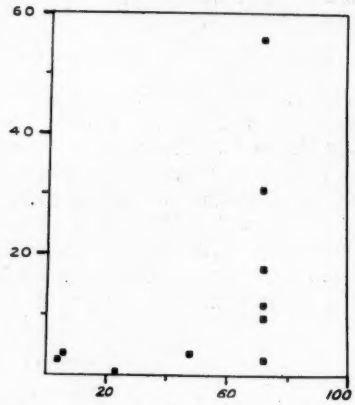


FIG. 2.

Number of mitoses seen in suprarenal after maximal single doses of Witte's peptone. Fig. 1, Expt. I; Fig. 2, Expt. II.

Ordinates; total number of mitoses in a pair of suprarenals (one section of each gland); abscissæ; time in hours after dosing. Each square denotes one mouse. When total count is the same for two or more mice, their number is shown by the area of rectangular blocks. The base of such a block shows number of mitoses; the midpoint of the base marks the time.

The phenomenon of "polar contouring" (Hoerr, 1931) was observed. This term denotes that cortical cells have convex inner borders and concave outer borders. The absence of lesions is a notable fact. Every gland was scrutinized for evidence of necrosis, hæmorrhage, and nuclear degenerations such as pyknosis, but none was found. No gland was even congested.

CORRELATION OF CLINICAL CONDITION AND PROLIFERATION.

Experiment 2.

The changes in temperature and general condition of 10 mice were studied after giving each of them an intraperitoneal dose of 7·5 mgm. of Witte's peptone per gm. body-weight, as in Experiment 1. To preclude any rise of

temperature from overcrowding alone, each animal was kept in a separate wooden box. All the mice were 6 months old, and with one exception females, none of which were pregnant.

The temperature was taken by inserting an ordinary clinical thermometer into the rectum. Readings were taken on each animal just before it was dosed, and then at intervals of about an hour for the next 7 hours. On the two days after that of the injection the temperature was taken morning and evening, and also just before the kill on the third morning. The general condition of each mouse was noted when the temperature was taken.

Four mice, depressed after the injection, became steadily worse until death in 4, 6, 23 and 47 hours thereafter. Their initial temperature ranged from 38·6° to 37·2° ; in 1·25 hours after the dose it was under 35°, and remained so until death.

The other 6 mice were killed about 72 hours after the injection. Excepting the 2 mice dead in 4 and 6 hours, each was weighed at death. The suprarenals from each were fixed in bichromate-chloride.

The numbers of mitoses found at different times are shown in Fig. 2. The absence of abnormal proliferation between 4 and 47 hours after injection, and its presence at 72 hours, are in accord with the findings in Experiment 1.

The observations on the 6 mice killed at 72 hours are summarized in Table I:

TABLE I.—*Changes in Mice Surviving Maximal Dose of Witte's Peptone, Killed in 72 hours.*

Mitoses.	Temperature, °C.	Clinical condition.	Loss of weight per cent.
55	Fell 1·8° ; normal in 3 hours	No sign of illness	2
30	< 35° for 7 hours ; normal in 24 hours	Ill in 3 hours, improving in 4 hours ; normal in 24 hours	0
17	Fell >2·4° ; normal in 5 hours	Slightly ill in 2 hours ; normal in 3 hours	8
11	Fell >2·7° ; normal in 7 hours	Rather ill in 3 hours ; normal in 7 hours	13
9	Fell >3·1° ; normal in 7 hours	No sign of illness	15
2	Fell >2·6° ; < 35° for 7 hours ; normal in 24 hours	Ill for 7 hours ; normal in 24 hours	10

CONTROLS.

Before concluding that the cortical proliferation, drop in temperature and other signs of illness were due to peptone, it was necessary to study the isolated effects of—

(1) The act of injection into the peritoneal cavity ; for a fall in body-temperature would follow injection of a cooler liquid, irrespective of its dissolved contents.

(2) The act of taking the rectal temperature; and—

(3) The act of swabbing the mouse's abdomen, before and after injection, with methylated spirit, which might by its evaporation cause a drop in temperature.

The results of these control procedures will be summarized together below.

(1) *Effect of Sodium Chloride.*

A solution containing 0·85 gm. of sodium chloride in 100 c.c. of distilled water was injected intraperitoneally into 10 mice, each mouse receiving the same volume of liquid per gm. body-weight as those in Experiments 1 and 2. The weight before and after experiment, temperature and general condition were noted as in Experiment 2. The animals were killed about 72 hours after dosing, the suprarenals being fixed in bichromate-chloride.

(2) *Effect of Taking Rectal Temperature.*

The rectal temperature of 10 normal mice was taken at intervals of about an hour for 7 hours. Next day these mice were used for studying the—

(3) *Effect of Swabbing with Methylated Spirit.*

The 10 mice were swabbed as for an injection 1 hour after the first reading, and their temperatures taken at intervals of about an hour for 7 hours.

Results.

No control animal showed any sign of illness. The injection of sodium chloride into 10 mice was followed in 1·25 hours by a fall in temperature in 8, the maximal fall being 0·9° C., *i. e.* just half the minimal fall after peptone, and by a rise in 2. Of 10 normal mice, 5 showed a fall not exceeding 0·3° C., 4 showed a rise, 1 no change, at 1 hour after the initial reading. Of the same 10 mice 0·75 hour after swabbing with methylated spirit, 6 showed a fall, not exceeding 0·5° C., 3 showed a rise, 1 no change. After sodium chloride 8 mice showed a loss of from 4 to 11 per cent. of their initial weight, 2 showing no change. Of the normals, excepting 1 that littered, 7 showed a loss between 2 and 11 per cent., 2 showing no change. After sodium chloride, with the unexplained exception of 1 mouse showing 18 mitoses, none showed more than 5. In the 4 normals killed and examined, only 1 showed 1 mitosis.

DISCUSSION.

The Cause of the Proliferation.

Before concluding that the cortical proliferation and changes in clinical condition were due to the peptone administered, it was necessary to exclude the effects of the experimental procedures themselves. It was found that no control animal showed any sign of illness. The maximal fall in temperature after injecting sodium chloride was just half the minimal fall after peptone. Therefore the marked fall in temperature observed after giving peptone cannot be attributed to the mere injection of liquid into the peritoneal cavity.

Swabbing with methylated spirit was shown to cause no significant change in temperature. Therefore the fall in temperature after peptone must have been caused by peptone.

Loss of Weight and Proliferation.

Considering the 6 mice in Experiment 2 killed at 72 hours after dosing with peptone, it is seen that the 3 mice showing least loss of weight showed most mitoses. Their average loss of weight was 3 per cent., and they showed an aggregate of 102 mitoses. The average loss of weight of the other 3 mice was 13 per cent., and they showed an aggregate of 22 mitoses. This demonstrates that the degree of cortical proliferation varies inversely with loss of weight, and, since loss of weight is a measure of illness, that the mice least upset by the peptone showed the most active mitotic response.

Temperature, General Condition, and Proliferation.

It was shown that No. 55 showed the least disturbance of temperature, which also returned to the normal sooner than in the other mice. This mouse, moreover, showed no signs of illness whatever. No. 17, although showing a marked fall in temperature, was the next to recover, and only once showed slight signs of illness. These results show that cell division was most active in the cortex of those mice showing the least departure from the normal temperature and general condition. The harmony of this conclusion is marred by No. 30, which was rather ill for 7 hours after the injection, with a temperature below 35° C.

The Nature of the Proliferation.

Cell division is abnormally active in the suprarenal cortex in two types of condition. The first type is that in which lesions such as hæmorrhage and necrosis have been produced. The proliferative activity following such lesions may be termed " regenerative mitosis." Most of the reported instances of experimentally induced mitosis are clearly of this kind. The second type of condition is that in which no antecedent lesion is discoverable to account for the mitosis. Such proliferation seems to express the response to a functional demand, and may be provisionally termed " functional mitosis." Examples of functional mitosis were reported by Deanesly (1931), in the most detailed study of suprarenal enlargement so far made.

As noted, no lesions were found after peptone, hence the mitosis observed cannot be regarded as regenerative. The facts that the mitoses were most frequent in the germinative layer of the cortex, *i. e.* at the junction of the " glomerular " and " fasciculate " zones, and that they were distributed fairly evenly round this layer, suggest that they indicate the response of the cortex to a demand for increased function. This view is supported by the clinical evidence already discussed.

The Frequency of Spiremes.

One peculiarity of cell division was noticed in all the glands examined, viz. the great frequency of spiremes, and the rarity of monasters and diasters. Attention was first drawn to this phenomenon apparently by Kolmer (1918), who concluded that the prophase lasts a long time in the cells of the suprarenal cortex. It was described in detail by Hoerr (1931), studying the guinea-pig suprarenal. He found that the phases of mitosis in order of decreasing frequency were prophase, metaphase, anaphase and telophase, these two phases probably never constituting more than 10 per cent. of the total number of mitoses. From the differential count of mitoses given under Experiment 1, it was found that the anaphase and telophase (diasters and dispiremes) formed 73 per cent., the prophase (monospiremes and monasters) 27 per cent. The metaphase was not seen. The significance of these percentages is doubtful because of two sources of error. First, it is possible that some spiremes reckoned as monospiremes may have been members of dispiremes, whose second members were not visible in the section. Second, some spiremes classed as dispiremes may really have been adjacent monospiremes, two adjacent cells chancing to be about to divide.

SUMMARY.

1. Intraperitoneal administration to mice of Witte's peptone in maximal doses was followed by abnormal proliferation in the suprarenal cortex.

2. Mitosis first became appreciable 56 hours after dosing, maximal between 71 and 82 hours thereafter, and was declining at 97 hours.

3. Mitoses were most numerous in the outer part of the cortex. In its inner part they were very rare.

4. Dispiremes far outnumbered the other phases of mitosis, which, in order of decreasing frequency, were monospiremes, monasters and diasters.

5. Mitosis was as a rule most active in mice showing the least departure from the normal temperature, general condition and weight.

6. Both morphological and clinical evidence supports the view that the mitoses seen after peptone are probably an indication of active function in the suprarenal cortex.

This investigation was suggested by Prof. J. Shaw Dunn, whose constant interest and advice I gratefully acknowledge.

REFERENCES.

Deanesly, R.—(1931) *Amer. J. Anat.*, **47**, 475.
Dunn, J. S.—Unpublished observations.
Hoerr, N.—(1931) *Amer. J. Anat.*, **48**, 139.
Kolmer, W.—(1918) *Arch. mikr. Anat.*, **91**, 51 (cited by Hoerr).

THE PRODUCTION OF TOXIN BY *C. DIPHTHERIÆ*. I: ENERGY SOURCES.

C. G. POPE.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

Received for publication April 13th, 1932.

THE Medical Research Council's Monograph on Diphtheria (Andrewes *et al.*, 1923) dealt with the production of toxin by *C. diphtheriæ* in an exhaustive manner, and supplemented the observations already reported by various workers by a series of special experiments designed to thrown more light on certain points. In spite, however, of the large amount of work which has been carried out, it is not yet possible to describe the mechanism by which the organism produces toxin.

The routine production of diphtheria antitoxin calls for large volumes of diphtheria toxin of suitable value. In the past, toxin was used which would now be considered of little value, but there is a growing tendency to use much stronger toxin at the present time. We have been successful in improving the medium used for toxin production at these laboratories, and for several years have discarded toxin having a value, as measured by the flocculation method of Ramon (1922), of less than 20 Lf units (the term " Lf " being that defined by Glenny and Okell, 1924). Of the total toxin produced, the proportion discarded has been small. The strongest toxin which we have produced had an Lf value of 50 units per c.c. (Pope, 1931). This value has since been obtained on two occasions with medium entirely different in composition, and we have produced many litres of toxin having an Lf value of over 40 units per c.c. The improvements which have been made in media designed for toxin production have been empirical, and not in any way based on a real knowledge of the manner in which toxin is produced by the organism. Much has been written on the necessity of using a suitable peptone, or on the question whether beef, veal or other meat provides the most suitable infusion, but little attention seems to have been given to the provision of a suitable energy source for the growing organism. It is in this direction that we have been successful in improving the toxin-producing value of our medium. Nevertheless, the advances which have been made in this empirical manner would probably be small as compared with those which might be expected if we had a more complete knowledge of the metabolism of the organism. In any attempt to gain such knowledge, it is obviously desirable to start with a medium whose composition is known, but unfortunately *C. diphtheriæ* is not an organism which grows with any degree of readiness on synthetic medium. Maver (1930, 1931) has, however, succeeded in growing the organism on synthetic medium, and moreover, has shown that some toxin is produced under these conditions. Unfortunately the amount of toxin produced was very small

(approximately 1/10 Lf unit per c.c. in the strongest toxin reported) and many months of work were necessary before the strain could be induced to grow with even moderate rapidity on these synthetic media. Wadsworth and Wheeler (1928) have attacked the problem in a different manner. They found it possible to produce a medium which contained peptone, but in which the meat infusion was replaced by known chemical substances, and on this they succeeded in producing toxin. This represented a big advance, because it was formerly held that growth of *C. diphtheriæ* with toxin production could not be obtained in peptone water in the complete absence of meat infusion. Davis and Ferry (1919), after a careful study of toxin production, came to the conclusion that it was impossible to obtain growth unless 0·2 per cent. of meat infusion was present, and that at least 10 per cent. of meat infusion was necessary for toxin production. The elimination of the meat infusion and its replacement by known chemical substances in the medium of Wadsworth and Wheeler reduces the number of variable components, although unfortunately the peptone still remains as one of the most unknown and variable factors concerned in the production of toxin. Wadsworth and Wheeler realized, however, the importance of having a suitable energy source present in the medium.

Our own interest in media of the semi-synthetic type arose from some unexpected results obtained while carrying out experiments with meat infusion. We have carried out a number of experiments on the production of toxin in semi-synthetic media, and these are reported in this paper. Unfortunately they have thrown little or no light on the mechanism of toxin production, but nevertheless we have produced media of this type which have given toxin of a value almost as high as that obtained with the more usual media produced from peptone and meat infusion or by the tryptic digestion of muscle.

EXPERIMENTAL.

An experiment showed that the material of value for toxin production present in meat infusion passed easily through parchment membranes. This experiment was later repeated with cellophane membranes and once again a similar result was obtained. The meat used in this experiment was veal, which after being freed from fat was minced, and then added to a 0·5 per cent. acetic acid solution, in the proportion of 2 lb. veal to each litre of acid. This was placed in a cellophane bag, and allowed to dialyse against an equal volume of acid solution for four days at 0° C. At the end of this period the liquid outside the bag was removed and to it was added 2 per cent. Difco proteose peptone, after which it was adjusted to pH 8·0. The mixture of meat and acid inside the bag was heated to 80° C. to coagulate protein and filtered. A similar amount of peptone was added to this filtrate, followed also by adjustment to pH 8·0. There was practically no change in the volume of the external liquid during dialysis. As a control medium we prepared a 2 per cent. peptone solution to which was added phosphates and 0·2 per cent. glucose, and this was adjusted to the same pH as the other portions. These three batches of medium were sterilized by filtration through Berkefeld candles, being distributed in 200 c.c. quantities in litre bottles. After incubation to check

sterility they were inoculated with Park 8 strain directly off Loeffler slopes and grown at 34–35° C. for 5 days. The results of this experiment may be summarized as follows :

	Cultures.	Lf per c.c.		
		Average.	Maximum.	Minimum.
Meat infusion—				
Inside dialyser	10	25	29	23
Outside ,,	9	23	25	21
Control medium—				
(No infusion)	9	14·5	17	11

The production of toxin of the strength shown in the control medium was surprising, as at this time we were not aware of Wadsworth and Wheeler's results, and accepted the generally held view that toxin could not be produced in the absence of meat infusion. From this first experiment we have continued an investigation of toxin production in semi-synthetic media, and have almost without exception confined our work to media in which Difco proteose peptone formed the sole source of nitrogen. The carbon or energy sources, on the other hand, have been varied in an attempt to find what constitutes the best material for the purpose.

Glucose, Fructose and Galactose as Carbon Sources for Toxin Production.

These sugars were used at a concentration of 0·2 per cent. in a peptone medium prepared in the following manner :

$MgSO_4$	0·2 gm.
$CaCl_2$	0·1 ,,
Na_2HPO_4	1·0 ,,
K_2HPO_4	1·0 ,,
Glacial acetic acid	3·0 ,,
Peptone (proteose)	20·0 ,,
Water	1000·0 c.c.

Adjusted to pH 8·0 with NaOH and filtered.

In each case the medium was sterilized by filtration through Berkefeld candles, 200 c.c. quantities in litre bottles being used as before. In no case was the medium heated in any way during preparation. All bottles were inoculated direct from Loeffler slopes using Park 8 strain, and no attempt was made at preliminary acclimatization to the medium. No difficulty was experienced in obtaining rapid film formation under these conditions. A control medium containing no carbohydrate was included in this series. The results obtained were :

	Cultures.	Lf per c.c.		
		Average.	Maximum.	Minimum.
Control medium—				
(no carbohydrate)	12	4·8	8	1
Glucose, 0·2 per cent.	11	18	21	14
Fructose, 0·2 ,,	12	19	23	26
Galactose, 0·2 ,,	9	18	23	9

The production of toxin in the control medium was probably due to the utilization of the acetate as an energy source, but acetate by itself forms a poor energy source, and is of more value when combined with other materials more easily utilized.

Organic Acids Used Alone and in Combination with Carbohydrates.

Wolf (1922) has shown that succinic and malic acids are rapidly attacked by *C. diphtheriæ*, with the production of an alkaline reaction in the medium. When certain carbohydrates are used in the medium, the amount which it is possible to add is limited owing to marked acid production. The acid production may be sufficiently large to stop further growth of the organism, and in the absence of reversal of the reaction to the alkaline side practically no toxin is produced. The oxidation of organic acids has been shown by Ayers and Rupp (1918) to be an important factor in reversal of reaction for other organisms. It was therefore decided to investigate the action of a number of organic acids (in the form of sodium salts), both alone and combined with glucose, on toxin production. So far we have investigated succinic, malic, fumaric, maleic, lactic, acetic, citric and pyruvic acids. In these experiments the stock peptone previously described was used unless otherwise stated, and sterilization effected by Berkefeld filtration. Park 8 strain was used throughout, and inoculation made direct from Loeffler slopes without any attempt at acclimatization. All media were adjusted to pH 8·0. The results are shown in Table I.

Medium containing succinic acid as the energy source gave toxin of moderate strength with a rather high final pH in most cases. Generally the Lf value appeared better with the higher pH values than at those with a pH nearer what is usually considered optimal. On combining 0·2 per cent. glucose with the succinic acid, the final pH values were lower but toxin production was considerably improved. An increase in the amount of glucose to 0·5 per cent. led to very irregular reversal changes. Some bottles remained acid and gave practically no toxin while others showed delayed reversal, and in these the toxin production appeared to depend on the final pH value.

Similar experiments were carried out with fumaric acid, but the concentration of organic acid was less than that used in the previous experiment, being 0·2 per cent. instead of 0·5 per cent. The results using the acid alone were somewhat variable, and apparently unconnected with the final pH. When, however, an addition of 0·2 per cent. glucose was made, two points of some interest were noticed. In spite of the added glucose the final pH values were higher than was the case with fumaric acid alone, and also there was a very marked increase in the amount of toxin produced. An increase in the concentration of glucose to 0·5 per cent. led to excessive acid production, and all the cultures had a pH value below 7·0 at the end of four days' growth. They contained no toxin which could be detected by the flocculation test even when the culture filtrates were blended with strong toxin (see Glenny and Okell, 1924).

The third acid used was malic acid, and the concentrations were as for fumaric acid. This acid proved to be much inferior for toxin production, as

TABLE I.

Succinic.						Fumaric.						Malic.					
A.		B.		C.		A.		B.		C.		A.		B.		C.	
Lf.	pH.	Lf.	pH.	Lf.	pH.	Lf.	pH.	Lf.	pH.	Lf.	pH.	Lf.	pH.	Lf.	pH.	Lf.	pH.
9	8·2	11	8·3	0<	7·0	5	8·4	23	8·7	0<	7·0	3	8·5	9	8·8	0<	7·0
10	8·5	18	8·7	2	7·0	5	8·6	23	8·7	0	,,	0	8·2	10	8·8	0	,,
12	8·8	25	8·7	17	8·7	5	8·4	25	8·6	0	,,	2	8·6	12	8·8	0	,,
16>	9·0	21	8·7	2<	7·0	9	8·5	23	8·9	0	,,	2	8·5	12	8·8	0	,,
17>	9·0	20	8·7	4	7·2	9	8·4	25	8·8	0	,,	2	8·5	11	8·8	0	,,
19>	9·0	23	8·7	15	8·7	9	8·5	31	8·8	0	,,	2	8·3	12	8·8	0	,,
21>	9·0	22	8·8	9	8·0	11	8·4	24	8·8	0	,,	2	8·3	14	8·8	9	8·6
19	8·9	21	8·8	8	8·0	13	8·6	25	8·8	0	,,	2	8·6	..		0<	7·0
21>	9·0	..		8	7·7	11	8·6	..		..		2	8·2	..		0<	7·0
						12	8·6										

Succinic: A, Stock peptone + 0·5 per cent. succinic acid. B, as A, but with 0·2 per cent. glucose. C, as A, but with 0·5 per cent. glucose.

Fumaric: A, Stock peptone + 0·2 per cent. fumaric acid. B, as A, but with 0·2 per cent. glucose. C, as A, but with 0·5 per cent. glucose.

Malic: A, Stock peptone + 0·2 per cent. malic acid. B, as A, but with 0·2 per cent. glucose. C, as A, but with 0·5 per cent. glucose.

can be seen from the results in the table. Without glucose, practically no toxin was produced, and even with 0·2 per cent. glucose the toxin values were lower than those previously recorded for a 0·2 per cent. glucose medium. With the higher concentration of glucose all cultures except one had an acid reaction when harvested. In this for unknown reasons reversal had occurred, but the toxin value was low. From this experiment it would appear that malic acid is unsuitable for toxin production, although it is capable of promoting growth in the peptone medium.

Acetic and lactic acids.—These acids were added as sodium salts to the stock peptone medium to give concentrations of 0·2, 0·4, and 0·6 per cent., and the media then compared in respect to the growth of *C. diphtheriæ*. Each batch of medium was adjusted to pH 7·8. Acetic acid gave good growth at 0·2 per cent., but at higher concentrations growth was poor. On the other hand, lactic acid gave growth which increased with the acid content, and was very heavy at 0·6 per cent. Further experiments were then made, using lactate or acetate at a concentration of 0·6 per cent. in stock peptone, and additions of glucose from 0·1 to 0·5 per cent. From this experiment it was found that provided the concentration of glucose did not exceed 0·3 per cent. for acetate and 0·2 per cent. for lactate, the medium first became acid followed by normal reversal. Higher concentrations of glucose than those indicated led to acid production and no alkaline reversal followed.

Lactate was later tried both alone and combined with glucose, but only toxin of low value was obtained, in spite of the fact that very heavy growth could be obtained with lactate-containing medium. Some experiments were also carried out on growth-rate using a mixture of equal weights of lactic, acetic, succinic and fumaric acids ; when it was added to stock peptone to give a total acid concentration of 0·5 per cent. growth was very rapid and heavy. This rate diminished as the total concentration of acids was reduced, until at 0·05 per cent. growth was extremely slow. However, given sufficient time, the total amount of growth eventually obtained was of the same order as for the higher acid concentration. Thus it would appear that although the rate of growth may be determined by the concentration of suitable energy sources present, the total amount of growth is dependent on other factors.

Maleic acid—Maleic acid either alone or combined with glucose appeared unsuitable for toxin production. It is of interest that one unsaturated acid (fumaric) should prove so useful, while its isomer (maleic) should have an opposite effect on toxin production.

Pyruvic acid.—This acid also proved to have little value either alone or combined with glucose.

Citric acid.—No evidence could be found that this acid was utilized by *C. diphtheriæ*.

The above results were obtained on small volumes of broth, but a large number of experiments have been carried out on 500 or 1000 c.c. quantities treated under the same conditions as routine medium. It would involve too much space to consider these in detail, but the results of three batches of special importance will be given. In these the stock peptone mixture was common to the three batches, and the amount of sodium acetate was increased to 0·5 per cent. The other details are given below.

Batch I :

Stock acetate peptone + 0·2 per cent. glucose.
Sterilized by Berkefeld filtration.
Litre quantities in 4-litre bottles grown for 10 days at 35–36° C.
Total number of bottles, 15. Lf units per c.c. : max. 15, min. 7, av. 11·6.
Harvesting pH values : max. 8·7, min. 8·0.

Batch II :

Stock acetate peptone + 0·3 per cent. glucose.
Sterilized by Berkefeld filtration.
Litre quantities in 4-litre bottles grown for 10 days at 35–36° C.
Total number of bottles, 7. Lf units per c.c. : max. 15, min. 0, av. 4.
Harvesting pH values : max. 8·3, min. below 7·0.

Batch III :

Stock acetate peptone + 0·15 per cent. glucose + 0·3 per cent. maltose.
Sterilized by Berkefeld filtration.
Litre quantities in 4-litre bottles grown for 10 days at 35–36° C.
Total number of bottles, 4. Lf units per c.c. : max. 28, min. 25, av. 27.
Harvesting pH values : max. 8·3, min. 7·8.
Sterilized by autoclaving.
Total number of bottles, 8. Lf units per c.c. : max. 11, min. 7, av. 8.
Harvesting pH values : max. 8·2, min. 8·0.

Consideration of these results shows several points of interest. Where glucose is added to the medium, the amount which may be added is of course limited owing to acid production, but even where the amount is " border-line " and a proportion of the bottles reverse, there is no evidence that the largest amount of glucose which just allows reversal gives a stronger toxin than a smaller amount. The maximum toxin value produced at either 0·2 or 0·3 per cent. glucose was 15 Lf units. In general it has been the experience that no advantage is to be gained by increasing the concentration of glucose above 0·1–0·2 per cent., unless special measures are taken to compensate acid production. The combination of sugars falls into another class of reversal control than indicated above. Experiments which have been carried out have shown that our strain of Park 8 produces little or no acid from maltose. (The results with maltose are given later.) It was therefore incorporated in Batch III, and at the same time the amount of glucose was reduced. In this batch we were unable to complete the sterilization of the medium by Berkefeld filtration on the day of preparation and the uncandled portion was sterilized by autoclaving (30 mins., 10 lb. pressure). In the candled portion toxin of high value was produced, whereas the portion sterilized by autoclaving gave toxin of a much lower value. This effect of heat sterilization has been very noticeable in work with semi-synthetic medium. In the further experiments to be recorded in this section we found it was impossible to carry out the sterilization by Berkefeld filtration, owing to the large number of subdivisions comprising each group, and it became necessary to employ heat sterilisation. This was reduced to a minimum, and throughout this series we used 10 mins.

autoclaving at a pressure of 15 lb., the bottles being removed from the autoclave as soon as possible and cooled fairly rapidly.

Toxin Production with Glucose Fumarate Media (Sterilized by Autoclaving).

Some results for media containing both glucose and fumaric acid have been given earlier in this paper. Further experiments were carried out with media containing a fixed amount (0·2 per cent.) of fumaric acid and glucose in concentrations varying from 0·05 to 0·4 per cent.

The results obtained in this medium when sterilization was carried out by autoclaving were entirely different from those for the candled medium. No bottles in the series had an Lf value higher than 8 units, and most bottles had a value of 6 to 8 units except at 0·4 per cent. glucose, where values as low as 0 were obtained. Except at the highest concentrations of glucose, the amount of this carbohydrate present was without effect on the amount of toxin produced. The changes in the pH during growth were marked. Concentrations of glucose up to 0·15 per cent. showed no acid phase, while from 0·2 to 0·4 per cent. acid was produced, the pH falling below the cresol red range. Reversal was fairly rapid except at a concentration of 0·4 per cent. glucose, where it became irregular. The pH changes were followed by incorporating 20 mgm. of cresol red into each litre of medium, experiments with both routine and semi-synthetic broths having shown this indicator to be without effect on toxin production.

Maltose Fumarate Media (Sterilized by Autoclaving).

A similar experiment was carried out with fumarate and maltose. With this carbohydrate there was no sign of any acid production during growth, and all bottles showed a reaction of approximately pH 9·0 after four days. There was a general tendency for the toxin values to increase with increasing concentration of maltose, but the maximum value was not high, being 12 Lf; minimum value 5 Lf units.

Galactose Fumarate Media (Sterilized by Autoclaving).

Media containing 0·2 per cent. fumaric acid and galactose in concentration from 0·05 to 0·4 per cent. were prepared. Again no acid production was obtained at any concentration of galactose, but the final pH values after four days' growth were remarkably consistent, the pH being 8·3 in almost all the bottles irrespective of galactose concentration. Nevertheless there was a definite increase in toxin values with increasing concentration of galactose, and at 0·4 per cent. the Lf value was 12–14 units.

Glycerine Fumarate Media (Sterilized by Autoclaving).

Hartley (1922) has shown that the presence of glycerine in trypsin preparations used for digest medium may lead to excessive acid production during the growth of *C. diphtheriæ*. With this experience in mind we tried glycerine

in combination with fumaric acid, using the concentrations previously described for the carbohydrates. There was no evidence of acid production, and judged by growth and toxin production there was little evidence that glycerine formed an available energy source for our substrain of Park 8 *C. diphtheriæ*. The maximum Lf value obtained in this series was 4 units per c.c. As this medium contained fumaric acid, the oxidation of which might have obscured any acid production from the glycerine, a further experiment was carried out with similar concentrations of glycerine but without fumaric acid. Growth was very poor, and there was no evidence of acid production.

DISCUSSION.

The results given in this preliminary paper deal with the value of a number of substances which have been studied as possible energy sources for *C. diphtheriæ*, and in particular for their effect on toxin production. We were able to obtain growth and moderate toxin with medium containing proteose peptone, together with other components all of known composition, and in this way could study the effect on toxin production of materials either singly or in combination. In this respect the observations reported in this paper differ from others except for those of Wadsworth and Wheeler (1928), who used a similar type of medium.

The addition of glucose to the usual type of infusion peptone medium has been suggested by many workers (Park and Williams, 1896; Theobald Smith, 1899; Locke and Main, 1928; Ramon, 1929; Hazen and Heller, 1931) for the purpose of increasing toxin production. In the method suggested by Theobald Smith, the meat infusion was deprived of fermentable substances by inoculation with *B. coli*, and glucose added in a definite concentration later. During the fermentation by *B. coli* there was marked production of acids, and it is highly probable that the success of Smith's method depended on the presence of sodium salts of these organic acids in the final medium. Locke and Main added 0·1 per cent. glucose to their broth, and after reversal when the pH reached 7·8 they made a second addition of 0·1 per cent. glucose. The second addition of glucose was found by them to increase toxin production by 50 per cent., and they suggested that this method should be useful for routine production of toxin. The toxin which they produced had an Lf value of about 13 units per c.c.—a value far lower than that which can be attained on a routine scale using digest medium of the type described by Hartley (1922)—without the added complication of having to add glucose aseptically during growth. A similar criticism applies to the method of Hazen and Heller. They added glucose daily, and at the same time readjusted the pH to 8·0. Notwithstanding the laborious nature of the method their yields of toxin were low, being about 15 Lf per c.c. We are, however, able to confirm the value of the combination of glucose and maltose which they used, and in semi-synthetic medium using these two sugars we have produced both on the experimental and routine scale culture filtrates of 30 or more Lf units per c.c.

The results which have been given show that glucose, fructose or galactose at a concentration of 0·2 per cent. is capable of increasing toxin production in a peptone solution to which has been added inorganic salts and sodium

acetate. Some toxin was produced when the only energy source was the sodium acetate, but this material is not readily metabolized by the organism. With an addition of more easily utilized organic acids there is at once a marked increase in both growth and toxin production. Thus succinic acid gave toxin of the same order of strength as was obtained with the sugars. For some reason fumaric and malic acids appeared to be less easily utilized and gave lower value toxins. However, when they are combined with glucose, several points of interest arise. With succinic acid it will be noticed that there was a direct relation between the final pH and Lf value. Thus with a final pH of 8·2 the toxin was of low value, and only at pH values of 9·0 or more was high value toxin produced. From many experiments with succinic acid it may be said that a high pH value at harvesting is necessary before strong toxin is obtained. This is obviously not in agreement with the results of Bunker (1919), or of Locke and Main who found that the optimal reaction for harvesting was about pH 8·0. When succinic acid is combined with glucose, the acid production from the glucose is more marked than would be expected from the rapidity with which succinic acid alone was oxidized with the production of an alkaline reaction. With fumaric acid the final pH was of the order of 8·5 at the end of growth, whereas with 0·2 per cent. glucose added, the pH values were slightly higher (8·7–8·8). Using the sodium salts of certain organic acids, it appears to be possible to control, to some extent at any rate, the production of acid and the subsequent reversal, but this is only one method of controlling these changes, and other methods will be reported later.

All the semi-synthetic media containing sugars with which we have worked have been very sensitive to heat sterilization. There is no detectable change in growth-rate or in reaction changes in media sterilized by autoclaving, but nevertheless medium so sterilized is of little value for toxin production. Our results in this direction are in striking contrast with those of Wadsworth and Wheeler (1928), who failed to obtain toxin with semi-synthetic media sterilized by Berkefeld filtration, but did so after the medium had been heated. They suggested that calcium played some part, and that it was necessary to heat the peptone with phosphates and calcium in order to produce colloidal calcium phosphate in the medium. In our work we have obtained no direct evidence that calcium plays any special part in toxin production. They also use in their medium ammonium lactate ; we have found lactate to be of little value for toxin production, and further, the ammonium salts of acetic, lactic and succinic acids do not stimulate the growth of *C. diphtheriæ* on peptone water in the way we have found for the corresponding sodium salts.

In this work we have not acclimatized the strain to the medium, but have inoculated directly from Loeffler slopes. As recently as 1930 (Hewlett, 1930), the statement appears that " for good toxin production all authors agree that the culture should be acclimatized to the particular broth employed." Throughout our work either with routine or semi-synthetic medium we have found no evidence that anything is to be gained by acclimatizing the organism to the medium.

While most of our measurements have for convenience been made by means of the flocculation test, we have satisfied ourselves that such results agree with those obtained with animal tests, and there is no indication that the toxin

produced on semi-synthetic medium differs from that obtained from infusion containing medium.

A number of other substances have been studied as energy sources, but work on them is not yet complete, and it is hoped to deal with these later.

Finally there is the question of the substances supplied in the meat infusion generally used for toxin production. Are these merely organic acids and small amounts of carbohydrate, or is there present in addition some other substance having an influence on toxin production ? In spite of a large amount of work with semi-synthetic media, we have not succeeded in producing toxin with an Lf value much above 30 units per c.c., whereas with the same proteose peptone and veal infusion we have produced several hundred litres of toxin with an Lf value considerably above 30 units, and in one batch a value of 50 Lf units per c.c. was obtained. The ease with which high value toxin can be obtained when meat infusion is used suggests that other substances are present which play an important part in toxin production. Further experiments bearing on this point will be reported later.

SUMMARY.

(1) The material present in meat infusion which is necessary for toxin production passes easily through cellophane membranes.

(2) Toxin has been produced on semi-synthetic medium in which the peptone was the only substance whose chemical composition was unknown. A number of organic substances have been studied for their effect on growth and toxin production.

(3) The meat infusion may be replaced by—

(*a*) glucose, fructose or galactose,
(*b*) combinations of glucose and maltose,
(*c*) the sodium salts of certain organic acids,
(*d*) or combinations of these acids with glucose,

all of which are capable of giving toxin of fairly high value. It is thought, however, that there are other substances present in meat infusion which affect toxin production.

REFERENCES.

ANDREWES, F. W., *et al.*—(1923) ' Diphtheria.' London (H.M. Stationery Office).
AYRES, S. H., AND RUPP, P.—(1918) *J. Infect. Dis.*, **23**, 188.
BUNKER, W. M.—(1919) *J. Bact.*, **4**, 379.
DAVIS, L., AND FERRY, N. S.—(1919) *Ibid.*, **4**, 217.
GLENNY, A. T., AND OKELL, C. C.—(1924) *J. Path. Bact.*, **27**, 187.
HARTLEY, P.—(1922) *Ibid.*, **25**, 479.
HAZEN, E. L., AND HELLER, G.—(1931) *Proc. Soc. Exp. Biol. Med.*, **28**, 423.
HEWLETT, R. T.—(1930) ' System of Bacteriology,' **5**, 84. London (H.M. Stationery Office).
LOCKE, A., AND MAIN, E. R.—(1928) *J. Infect. Dis.*, **43**, 41.
MAVER, M.—(1930) *Ibid.*, **47**, 384; (1931) *ibid.*, **49**, 1.
PARK, W. H., AND WILLIAMS, A. W.—(1896) *J. Exp. Med.*, **1**, 164.
POPE, C. G.—(1931) *Lancet*, **2**, 402.
RAMON, G.—(1922) *C.R. Soc. Biol.*, **86**, 661; (1929) *C.R. Acad. Sci.*, **101**, 718.
SMITH, T.—(1899) *J. Exp. Med.*, **4**, 373.
WADSWORTH, A., AND WHEELER, M.—(1928) *J. Infect. Dis.*, **42**, 179.
WOLF, C. G. L.—(1922) *Biochem. J.*, **16**, 541.

THE PRODUCTION OF TOXIN BY *C. DIPHTHERIÆ*. II: EFFECTS PRODUCED BY ADDITIONS OF IRON AND COPPER TO THE MEDIUM.

C. G. POPE.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

Received for publication April 13th, 1932

In a previous paper (Pope, 1932) we described the results of experiments on the production of toxin in semi-synthetic media and the value of various substances as energy sources. During the course of these experiments certain observations were made on the effect of introducing into the medium small amounts of iron and copper, and these are reported in this paper. A special effect of copper in certain concentrations on the morphology of *C. diphtheriæ* has already been dealt with (Pope and Pinfield, 1932).

The medium used in this work had the following composition for the first two experiments, but was later modified by the addition of 0·2 per cent. succinic acid.

$MgSO_4$	0·2 mgm.
Na_2HPO_4	1·0 gm.
K_2HPO_4	1·0 ,,
Sodium lactate 50 per cent. solution	12 c.c.
Glucose	2·0 gm.
Cresol red	20 mgm.
Proteose peptone	20 gm.
Water	1000 c.c.

The medium was adjusted to pH 8·0 and sterilized by Berkefeld filtration.

Experiment 1.—The above medium was filtered into bottles of 500 c.c. capacity, each receiving 100 c.c. of medium. After a preliminary incubation to check sterility, the medium was inoculated with *C. diphtheriæ*, Park 8 strain. At the end of 24 hours' growth there was marked acid production, the pH being too low to read from the colour of the cresol red present in the medium. Incubation was continued for a period of 15 days, but there was never any indication of reversal, and no toxin was found in the filtrates.

Experiment 2.—The first experiment was repeated as closely as possible, except that iron in the form of ferric citrate was added to give a concentration of Fe^{+++} ions of 0·5 mgm. per litre of medium. The reaction changes in this medium were as follows: At the end of 24 hours' growth, the pH had fallen to 7·2 or less; at 40 hours there were slight signs of reversal, which became quite definite 4 hours later. After 66 hours the reaction was about pH 8·0

and quite uniform in all cultures. When harvested at the end of 4 days' growth, the pH value was 8·6. This group consisted of 8 bottles, and the average toxin value was 14 Lf units per c.c: minimum 14, maximum 15 units.

The medium was then modified by the addition of 0·2 per cent. succinic acid, in order to limit slightly the acid phase and to ensure that reversal occurred even without the addition of iron.

Experiment 3.—A batch of the modified medium was prepared, adjusted to pH 8·0, and filtered through paper. At this stage it was divided into two equal portions, to one of which ferric citrate was added to give 0·5 mgm. Fe^{+++} per litre. Both were sterilized by Berkefeld filtration, inoculated, etc., as in the previous experiments. The results are summarized below:

	Control. No iron.	With 0·5 mgm. iron per litre.
Growth	Moderate	Very heavy
Final pH	Average 7·8	8·6
Acid phase	Very marked	Very slight
Average Lf	7	17·5
Maximum Lf	11	19
Minimum Lf	4	16
Microscopic appearance	Short bacilli; no polar staining	Longer bacilli with marked polar staining

Further experiments were then carried out in a similar manner, but using increased amounts of iron, and the results of two typical experiments are given:

	Iron per litre of medium.			
	Nil.	0·5 mgm.	1·0 mgm.	2·0 mgm.
Average Lf	11	15	0·5	0
,, ,,	12	12	6	0·5

It will be noticed that in these experiments the control medium gave stronger toxin than was obtained in Experiment 3. This was probably due to the fact that reversal occurred more readily, but the matter will be dealt with later in the paper. The results do show, however, that any marked increase in the iron content diminished the toxin production. When the medium contained 2·0 mgm. iron per litre, the reversal phase was very rapid, the pH which at first fell to about 7·2, changed in less than 12 hours to pH 8 or more, and at the end of 4 days was more alkaline than pH 9.

At this stage the work was unavoidably delayed for a time, and when we were able to return to this particular point we had changed the method of sterilization from Berkefeld filtration to heat sterilization. This change eventually brought to light certain points which might otherwise have been overlooked.

Experiment 4.—In this experiment copper and iron were added to the medium. Sterilization, as stated above, was effected by autoclaving for 10 minutes at 15 lb. pressure. The cultures were grown for 4 days, and at the

end of growth the reaction had become somewhat alkaline (pH 8·8 to 9·0). The effect of added iron or copper on toxin production is shown below :

Mgm. per litre.		Lf units per c.c. Results on individual bottles.				
Cu^{++}.	Fe^{+++}.					
4·0	0·0	21	20	21	21	19
3·2	0·4	6	7	5	6	3
2·4	0·8	3	1	4	4	2
1·6	1·2	0	0	2	0	2
0·8	1·6	4	2	2	2	4
0·0	2·0	0	2	2	2	2

Experiment 5.—On obtaining these results we again made experiments with this medium and varying amounts of iron, but failed to obtain the enhancement of toxin with low concentrations of the order of 0·5 mgm. per litre. Various concentrations of copper alone were also studied, and in addition two peptones—(*a*) Difco proteose, and (*b*) Parke Davis. The results are summarized in Table I.

TABLE I.

Cu^{++} or Fe^{+++} added per litre.	Average Lf (approx.) 5 bottles in each group. Parke Davis peptone.	Difco proteose peptone.
Cu^{++} 8 mgm.	11 (8–13)	11 (7–13)
6 ,,	11 (9–14)	10 (9–11)
4 ,,	6 (5–8)	10 (9–12)
3 ,,	6 (3–8)	10 (7–11)
2 ,,	5 (2–8)	10 (7–11)
1 ,,	3 (2–4)	10 (9–13)
Control	2 (0–2)	9 (8–11)
Fe^{+++} 0·2 mgm.	1·5 (0–2)	4 (3–5)
0·4 ,,	1·5 (0–3)	1 (0–2)
0·6 ,,	1·0 (1–2)	0 (0–1)
1·0 ,,	1·5 (0–3)	0 0
1·5 ,,	1·5 (0–2)	0 0

The figures in brackets refer to the highest and lowest Lf value found in each group.

On reviewing our methods it was realized that the method of sterilization might have some effect, and we therefore carried out a further experiment, which is now described.

Experiment 6.—For this experiment we prepared a further batch of medium, and to it added either iron or copper as desired. Each portion of medium was then divided into two parts, one of which was sterilized by Berkefeld filtration, and the other by autoc'aving. Only three bottles were included in each group, as we wished to complete the preparation of the medium and its final sterilization during one day, to avoid any effects which might result from bacterial contamination. One bottle from each group was inoculated from a

Loeffler slope of Park 8 strain, and when film formation was complete it was used to sow the remaining two bottles A and B of its group. The results are summarized in Table II.

TABLE II.

Cu^{++} or Fe^{+++} added per litre.	Sterilized by— Autoclaving. Lf. Bottle. A.	Autoclaving. Lf. Bottle. B.	Autoclaving. pH. Bottle. A.	Autoclaving. pH. Bottle. B.	Berkefeld filtration. Lf. Bottle. A.	Berkefeld filtration. Lf. Bottle. B.	Berkefeld filtration. pH. Bottle. A.	Berkefeld filtration. pH. Bottle. B.
Cu^{++} 8·0 mgm.	10	14	8·4	8·5	12	13	8·4	8·5
6·0 ,,	9	9	8·4	8·3	10	10	8·3	8·3
4·0 ,,	13	12	8·4	8·4	15	9	8·4	8·3
3·0 ,,	9	10	8·4	8·4	11	12	8·3	8·3
2·0 ,,	8	12	8·4	8·4	9	9	8·3	8·3
1·0 ,,	11	11	8·4	8·4	9	12	8·3	8·3
Control	9	11	8·4	8·4	3	12	7·5	8·0
Fe^{+++} 0·2 mgm.	2	5	9·0	9·0	7	11	8·4	8·4
0·4 ,,	4	1	9·0	9·0	12	11	8·4	8·5
0·6 ,,	0	1	9·0	9·0	6	7	8·4	8·4
1·0 ,,	1	6	9·0	9·0	10	5	8·4	8·8
1·5 ,,	3	3	9·0	9·0	5	9	8·5	8·5

DISCUSSION.

As far as we are aware the only studies on the influence of iron and copper on the production of toxin by *C. diphtheriæ* are those of Locke and Main (1930, 1931), who used medium prepared from peptone and meat infusion. They state that medium as usually prepared for production of diphtheria toxin contains about 0·001 to 0·004 mgm. of copper, and approximately one-tenth to one-twentieth that quantity of iron per c.c. (= 1 to 4 mgm. Cu/litre and about 0·1 to 0·4 mgm. iron/litre). Our own observations are roughly in agreement with the figures quoted by them for copper, but we find that nearly all the copper is present in the peptone, and that little, if any, is contributed by the meat infusion as prepared by us. In their medium they found that *C. diphtheriæ* produced with regularity a culture filtrate containing 600 to 800 m.l.d. per c.c. For comparison with our results we have converted their value in m.l.d. per c.c. into Lf units per c.c., the value being approximately 12 Lf, assuming that the ratio of toxin to toxoid in their material was similar to what we find in our culture filtrates. Substances which depressed the Cu^{++}/Fe^{+++} ratio were found by them to reduce toxin production, while the addition of 2·5 mgm. Cu^{++}/litre slightly increased it. For the experiments reported in this paper we worked with a semi-synthetic medium, designed to have a marked acid phase with slow reversal, so that we could study the effect on reversal of catalysts of the iron and copper type. The medium must therefore be considered as one unsuitable for general toxin production, but possessing special

features which have enabled us to study certain points in connection with the rate of reversal and its effect on the toxin produced.

The first and second experiments show that a small concentration of iron has a marked effect on the reversal change during growth. Whereas without added iron there was complete failure of reversal and no toxin was produced, with as little as 0·5 mgm. iron/litre, reversal proceeded normally, and there was moderate toxin production. Even with medium modified by the addition of a little succinic acid, the acid phase was very pronounced in the absence of added iron.

The amount of iron which can be added for the purpose of ensuring that reversal occurs is limited, otherwise there is a marked reduction in the amount of toxin found in the culture filtrates. From our experiments with iron it would appear that the amount added should not be greater than is necessary to secure a normal reversal, such that the reaction at the end of 4 days' growth is pH 8·4–8·8 for 100 c.c. quantities of medium in 500 c.c. capacity bottles.

These results for medium sterilized by filtration could not be repeated when heat sterilization was employed. Material sterilized by autoclaving appeared not to require the addition of iron, as reversal took place in a normal manner. An investigation of our results for a number of experiments in which sterilization had been made by Berkefeld filtration, on medium which only produced a small amount of acid during the early stages of growth, revealed the fact that the first portions of medium to pass through the candle always showed a lower final pH than later portions. Thus, where the filtrate from one 10 × 2 in. Berkefeld was received in 100 c.c. quantities, the first 3 to 5 portions might show this phenomenon. We had attributed this difference to impurities derived from the filter, but now suggest that the filter-candle removes, possibly by adsorption, from the first portions filtered, some substance which has a large effect on the reversal changes during growth. Filter candles appear to vary in the extent to which they show this property, but the effect has been obtained both with Berkefeld N and Pasteur-Chamberland F filters.

The influence of the method employed for sterilization is clearly shown in the results of Experiment 6. The control medium without added iron or copper showed a marked acid phase ; there was complete failure of reversal in the first generation ; partial reversal in one bottle, and more complete reversal in the other for the second generation. With the autoclaved series containing iron, the final reaction was pH 9 or more, but with the candled series a less alkaline reaction was obtained. It cannot be assumed that the alkaline reaction in the autoclaved series had caused destruction of toxin—in other experimental work we have obtained a culture filtrate containing 51 Lf units per c.c. with a final pH 9·0. It is more probable, therefore, that the low toxin values were due to failure of production rather than to any destruction of toxin at a pH value of about 9.

The addition of copper to the medium employed for this work seems in some cases to have enhanced toxin production, but it appears to have little obvious effect on the reaction changes during growth, except that in Experiment 6 there was never any sign of " reversal failure " in the candled series containing copper. The influence of copper on the reversal phase is far less striking than that produced by smaller amounts of iron. A concentration of

8–10 mgm. Cu^{++} per litre in this medium was about the maximum amount which could be tolerated by *C. diphtheriæ* without reducing markedly the growth rate.

The effect of adding copper or iron to tryptic digest medium has been tried. An addition of iron to a concentration of 0·5 mgm./litre reduced toxin production by more than 50 per cent., while copper in amounts varying from a fraction of a milligramme to several milligrammes had little effect on the amount of toxin produced.

SUMMARY.

Working with a special semi-synthetic medium, it has been shown that small additions of iron have a large effect on the reversal changes during growth of *C. diphtheriæ*. While small amounts of iron may favour toxin production, larger amounts inhibit it.

The reversal changes may also be affected by the method used for sterilization, and it has been shown that where sterilization is by Berkefeld filtration, the candles appear to remove from the medium some substance which plays a part in normal reversal. Where this substance has been removed by filtration, the addition of iron or copper leads to reversal, iron being much more active than copper.

REFERENCES.

LOCKE, A., AND MAIN, E. R.—(1930) *J. Infect. Dis.*, **46**, 393.
POPE, C. G.—(1932) *Brit. J. Exp. Path.*, **13**, 207.
Idem AND PINFIELD, S.—(1932) *Ibid.*, **13**, 60.

INTRANUCLEAR BODIES IN THE LIVER-CELLS OF MICE.

G. M. FINDLAY.

From the Wellcome Bureau of Scientific Research, London.

Received for publication April 23rd, 1932.

As is now well known, many ultramicroscopic viruses are capable of giving rise to intranuclear inclusions in the tissues of susceptible animals. So common, in fact, is the association between acidophilic intranuclear inclusions and ultramicroscopic viruses, that certain workers believe that the finding of such intranuclear bodies is in itself almost conclusive evidence of the presence of ultramicroscopic viruses. Despite numerous investigations, however, little is yet known in regard to these intranuclear inclusions, for though they tend to arise in the nucleoplasm, they possess certain specific differences in morphology and in staining which are characteristic for each virus. In the absence of more accurate knowledge of these inclusion bodies, more especially in regard to their method of formation by ultramicroscopic viruses, it would seem to be premature to assert that their presence invariably denotes infection by an ultramicroscopic virus.

There are, in fact, many instances where acidophilic intranuclear inclusion bodies have been found, but no ultramicroscopic viruses have as yet been

demonstrated. In man, Goodpasture and Talbot (1921), Wilson and DuBois (1923), von Glahn and Pappenheimer (1925) and others have described intranuclear bodies in the internal organs in cases exhibiting no very definite symptoms. Hindle and Stevenson (1930) have found intranuclear bodies in the kidneys of monkeys in captivity and in sewer rats. Cowdry and Scott (1930) have described intranuclear inclusion bodies in the livers of dogs, while recently Thompson (1932) has found acidophilic intranuclear bodies in the salivary glands of rats, and Covell (1932) in the livers of monkeys.

In the present preliminary communication intranuclear bodies are described in the liver-cells of mice.

During the course of investigations on Rift Valley fever which have been described elsewhere (Findlay and Daubney, 1931; Findlay, 1932), intranuclear bodies of a somewhat different type to those usually associated with the presence of ultramicroscopic viruses were found in the liver-cells of certain mice which had been infected with Rift Valley fever. As Rift Valley fever virus itself produces intranuclear acidophilic inclusions in the livers of sheep, as shown by Daubney, Hudson and Garnham (1931), careful search was made for similar inclusions in the livers of mice infected with this virus. At first the bodies about to be described were thought to be due to infection with Rift Valley fever; an examination of apparently healthy control mice from the laboratory stock showed, however, that a certain number had exactly similar bodies in their liver-cells. As these control mice had been in the laboratory for some days, it was possible, though not probable, that these mice were also suffering from Rift Valley fever, perhaps in an attenuated form. This possibility was excluded by the following observations: Blood from these control mice injected into other normal mice immediately on their arrival in the laboratory failed to cause Rift Valley fever, while an examination of the livers of mice killed as soon as they reached the laboratory eventually showed that the stock of one London dealer comprised mice with these intranuclear bodies in the liver. This London dealer, X, kindly supplied me with samples of all the mice that he received from breeders, and on examining the livers of these mice, one strain from Clacton was found to exhibit intranuclear inclusion bodies in the liver-cells. Seven other pure-bred strains of mice which were also examined for the presence of these bodies gave negative results. In the Clacton strain, however, every mouse was found to be affected to a greater or less degree:

Source.	Number of mice examined.	Number of mice positive.	Number of mice negative.
Clacton	25	25	0
Chesterfield I	10	0	10
,, II	10	0	10
York	10	0	10
R.	10	0	10
Banbury	12	0	12
W.P.R.L. (A)	4	0	4
,, (B)	4	0	4
Total	85	25	60

As the strains examined were derived from a variety of sources throughout the country, it is obvious that these intranuclear bodies are by no means common in mice. Twort and Twort (1932), for instance, did not notice their presence in the examination of 60,000 experimental mice. The rarity of these bodies in the livers of mice is also shown by the following occurrence, which, through the kindness of Dr. S. P. Bedson of the London Hospital, I am able to quote. Although the tissues of a large number of mice had been examined in the course of experiments on psittacosis, no intranuclear bodies were found in the livers of mice till certain mice were inoculated with a Berkefeld filtrate obtained from the tissues of a fatal case of broncho-pneumonia in man. Two of the mice thus inoculated died, and in their livers were found intranuclear bodies identical with those found in the Clacton mice. Investigations showed that these mice had also been supplied to the London Hospital by the same dealer, X.

Recently in the course of an examination of the livers of certain wood-mice (*Apodemus sylvaticus*), obtained from near Oxford by the kindness of Mr. C. Elton, Director of the Animal Populations Bureau, Oxford, the same intranuclear bodies have been found in the liver of one apparently healthy mouse. This mouse had, however, lived in the laboratory for some weeks before its liver was examined, so that the possibility of a hypothetical infection cannot be entirely excluded.

CHARACTERISTICS OF THE INTRANUCLEAR BODIES.

As previously mentioned, intranuclear bodies have been found in the livers of all adult mice of the Clacton strain examined. The bodies, however, were not present in the livers of six newly born mice of this strain. Though the number of cells affected varies in different animals, as shown in the following table, it is always very small. Observations are now being made to determine whether the number of affected cells increases or decreases with age.

No. of mouse.	Number of nuclei examined.	Number of nuclei affected.	Percentage of nuclei affected.
1	500	1	0·20
2	600	4	0·66
3	600	4	0·66
4	500	4	0·80
5	500	4	0·80
6	600	5	0·83
7	600	5	0·83
8	600	7	1·16
9	500	20	4·00
10	600	25	4·16
Total . 10	5600	79	1·04

Affected nuclei as a rule are hypertrophied, as is seen in Figs. 1 to 5, which illustrate the appearance of the intranuclear bodies. The bodies vary from about 8 to 80 or 90μ in diameter. In shape they are round or oval, and as

a rule rest against the nuclear membrane. Not infrequently two or more bodies may be seen within the same nucleus. The bodies appear in many cases to be bounded by a definite membrane, and when small are completely filled with apparently homogeneous material, though occasionally vacuoles appear in their interior (Fig. 1). In somewhat larger bodies the interior may not be completely filled (Fig. 2), but contains a varying amount of finely granular material, which is sometimes collected into an inner body (Fig. 3). In still larger bodies, as in Fig. 4, the nuclear chromatin is crushed by the body against the nuclear membrane until finally, as in Fig. 5, there is only a membrane filled with acidophilic granular material, while chromatin has disappeared from the nucleus. The intranuclear bodies may be recognized after treatment of the tissues by all the usually employed fixatives—formol-saline, Zenker with or without acetic acid, Bouin's fluid and corrosive formol. They are well seen when stained by hæmatoxylin or hæmalum and eosin, methylene-blue and eosin, phloxine and methylene-blue, hæmatoxylin and acid fuchsin. Zenker's fluid followed by hæmatoxylin and eosin has given the best results; in such preparations the smaller bodies stain a purplish red, but in the larger the inner material stains a brick red. With phloxine a very similar colour is produced, while with Giemsa's stain a purple-red tint is obtained. Tissues stained by Feulgen's technique show that the smaller bodies give a faint reaction for chromatin, while even in the larger bodies there is a very faint reaction, the internal structure staining a rose pink, in contradistinction to the dark aster purple of the chromatin. Very rarely there are one or two tiny basophilic bodies in the acidophilic granules similar to those described by Cowdry (1930) in Virus III inclusions. The intranuclear bodies have so far been found only in the liver-cells.

THE NATURE OF THE INTRANUCLEAR BODIES.

The morphology and the staining properties of the bodies suggest that in all probability they are hypertrophied nucleoli. Unfortunately little is known in regard to the nucleoli of the liver-cells as compared with those of epidermal and nerve-cells. Cowdry and Kitchen (1930), however, have recently studied the nucleoli of the liver-cells in monkeys in relation to the nuclear changes produced by the virus of yellow fever. They have shown that in monkeys the nucleoli of the liver-cells are amphoteric, a core of oxyphilic material, in reality basic material, being covered by a thin layer of chromatin or acidophilic material. As the nucleolus enlarges the layer of chromatin becomes attenuated. The nucleolus in the liver-cells of the mouse is also amphoteric. The smaller intranuclear bodies might, therefore, reasonably be interpreted as hypertrophied nucleoli in which the basophilic outer covering has become attenuated; the larger bodies, however, with their granular acidophilic material,

Liver-cells of "Clacton" mice to illustrate the development of the intranuclear bodies; fixed Zenker; stained Mayer's hæmalum and eosin.

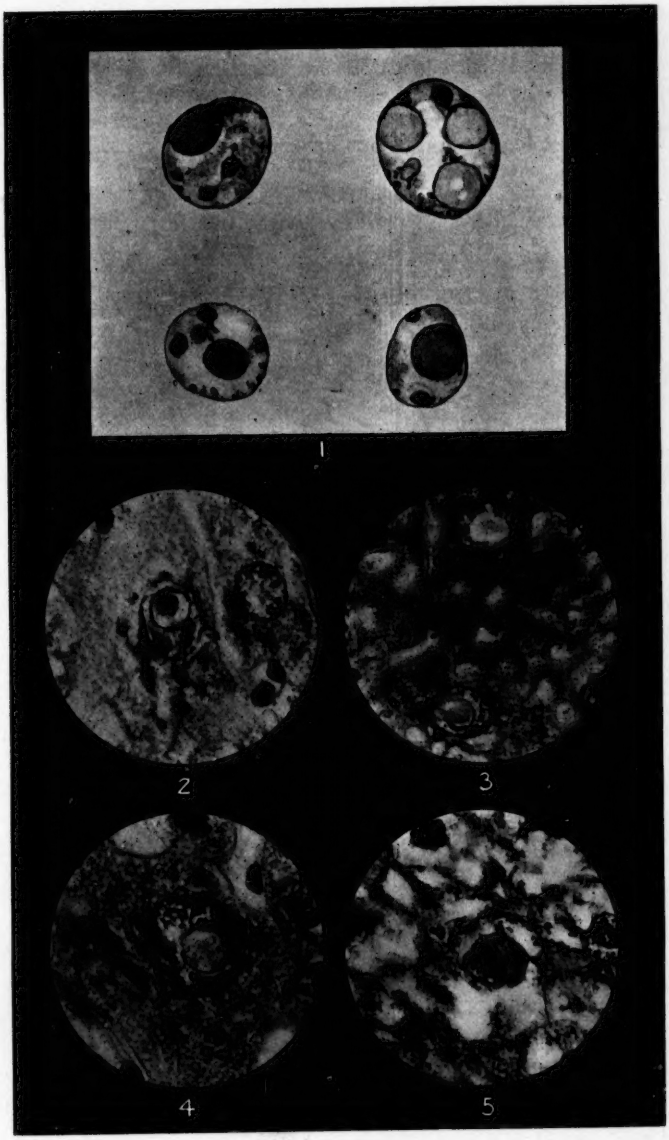
FIG. 1.—Early stages in the intranuclear bodies. Camera lucida drawing × 2000.

FIG. 2.—Intranuclear body with acidophilic material collected into an inner body. × 1000.

FIG. 3.—Intranuclear bodies in two adjacent liver-cells. × 1000.

FIG. 4.—Intranuclear body in an hypertrophied nucleus. × 1000.

FIG. 5.—Intranuclear body with granular acidophilic material completely filling the nucleus. × 1000.


1
2
3
4
5

differ considerably from typical hypertrophied nucleoli—those, for instance, figured by Cowdry and Kitchen as occurring in the human liver in yellow fever and acute yellow atrophy.

Sections of affected livers have now been submitted to a number of cytologists, and though many possible explanations for these bodies have been suggested, the general consensus of opinion is that their appearance differs from that of true nucleolar hypertrophy. Unfortunately the behaviour of the nucleolus under varying pathological conditions has received very little attention, and even under normal conditions no adequate explanation of its functions is forthcoming. The old view that the nucleolus was merely a refuse for waste products in the nucleus has many drawbacks, but has not been superseded by any more satisfactory explanation.

Possible explanations to account for a nucleolar hypertrophy of the liver-cells in this one strain of mice which suggest themselves are :

(1) A metabolic disorder.

(2) A Mendelian variation.

(3) An infection of a low order of pathogenicity.

(1) So far as can be judged from general appearances, death-rate and histological appearances of the organs, the Clacton mice were in no way abnormal except for the liver changes. Nucleolar hypertrophy in the liver in man has so far been associated only with gross pathological changes.

(2) If the appearances in the liver are due to a mutation it is not present at birth, nor has it been possible to find any record of a mutation affecting only the nucleoli of one organ.

(3) If due to an infection, protozoal and bacterial infection are improbable. No protozoa were found in sections of any organs, nor could bacteria be demonstrated in the tissues by Gram's stain or by Murray's thionin blue, Van Gieson method. On the other hand, the possibility of infection by an ultramicroscopic virus cannot be so easily excluded. A number of virus infections of low pathogenicity, almost saprophytic in character, are known in rodents, such as Virus III infection in the testicles of rabbits and salivary gland disease of guinea-pigs, while the intranuclear bodies of the salivary glands of rats recently described by Thompson (1932) are also probably due to a virus, as are the intranuclear bodies in the kidneys of sewer rats and monkeys (*M. rhesus*), described by Hindle and Stevenson (1930), and the intranuclear inclusions found by Covell (1932) in monkeys.

As a working hypothesis the probability of a virus infection of low pathogenicity has been adopted, and experiments have been carried out to test the possibility of transmitting the condition.

EXPERIMENTS ON TRANSMISSION.

Six mice of the Banbury strain were biopsied and a portion of liver removed for histological examination. The livers having been shown to be free from intranuclear bodies, the mice were inoculated intra-peritoneally with 0·5 c.c. of a suspension of the livers of Clacton mice, ground up in buffered broth at pH 7·2 to form approximately a dilution of 1 in 10. Bacteriologically the liver suspension was sterile. As a control, six other Banbury mice were inoculated

with a similar suspension of the livers of Chesterfield I mice. These mice were kept in a box next to the mice inoculated from the Clacton strain, and received the same food and treatment as the experimental mice. At intervals of ten days after inoculation two mice from each series were killed and the livers examined for intranuclear bodies.

Interval between inoculation and examination.	Strain.		Strain.	
	Banbury.	Clacton.	Banbury.	Chesterfield I.
10 days	+	−	−	−
20 ,,	+	+	−	−
30 ,,	+	+	−	−

While the Banbury mice inoculated with liver from Chesterfield I mice were all negative, five out of the six Banbury mice were positive.

An emulsion of the livers of the two infected mice killed twenty days after inoculation was made as before in broth, centrifuged to throw down gross particles and passed through a British Berkefeld filter. The filtrate, which was bacteriologically sterile, was inoculated intra-peritoneally in doses of 0·5 c.c. into six Banbury mice which had been shown to be negative by previous biopsies. Two mice were killed, as before, on the 10th, 20th and 30th days after inoculation. The mice killed on the 10th day after inoculation were negative, but one of those killed on the 20th day and both those killed on the 30th day showed the presence of intranuclear bodies in the liver.

Further experiments are in progress. In view, however, of the extended use of mice in the study of many virus infections it has seemed best to publish these preliminary observations, as the finding of acidophilic bodies in the liver-cells of injected mice may only too easily lead to fallacious conclusions.

CONCLUSIONS.

Intranuclear bodies have been found in the liver-cells of a strain of apparently normal mice. The appearances of these bodies are described, and evidence is brought forward to suggest that they are caused by a filterable virus of low pathogenicity.

REFERENCES.

Bedson, S. P.—(1931) Personal communication.
Covell, W. P.—(1932) *Amer. J. Path.*, **8**, 151.
Cowdry, E. V.—(1930) *Arch. Path.*, **10**, 23.
Cowdry, E. V., and Kitchen, S. F.—(1930) *Amer. J. Hyg.*, **11**, 227.
Cowdry, E. V., and Scott, G. H.—(1930) *Arch. Path.*, **9**, 1184.
Daubney, R., Hudson, J. R., and Garnham, P. C.—(1931) *J. Path. Bact.*, **34**, 543.
Findlay, G. M.—(1932) *Trans. Roy. Soc. Trop. Med. Hyg.*, **25**, 229.
Idem and Daubney, R.—(1931) *Lancet*, **2**, 1350.
Goodpasture, E. W., and Talbot, F. B.—(1921) *Amer. J. Dis. Child.*, **21**, 415.
Hindle, E., and Stevenson, A. C.—(1930) *Trans. Roy. Soc. Trop. Med. Hyg.*, **23**, 327.
Thompson, M. J.—(1932) *J. Infect. Dis.*, **50**, 162.
Twort, J. M., and Twort, C. C.—(1932) *J. Path. Bact.*, **35**, 219.
Von Glahn, W. C., and Pappenheimer, A. M.—(1925) *Amer. J. Path.*, **1**, 445.
Wilson, J. R., and Dubois, R. O.—(1923) *Amer. J. Dis. Child.*, **26**, 431.

THE TRANSMISSION OF LOUPING ILL TO MONKEYS.

G. M. FINDLAY.

From the Wellcome Bureau of Scientific Research, London.

Received for publication April 25th, 1932.

LOUPING ill, a disease of sheep especially prevalent in the border counties of England and Scotland, is now known, as a result of the work of Pool, Brownlee and Wilson (1930), to be due to a filterable virus with neurotropic properties. The disease was transmitted to mice and less certainly to rats by Greig, Brownlee, Wilson and Gordon (1931), who briefly described the lesions as being of the meningo-encephalo-myelitic type. Alston and Gibson (1931) also transmitted the virus to mice, but failed to infect rats, guinea-pigs and rabbits. Hurst (1931), in a recent communication, has shown that in mice the lesions are of the nature of a diffuse encephalo-myelitis with mild meningeal involvement, thus resembling the changes present in sheep which were described by M'Gowan and Rettie (1913) as consisting of an infiltration with small round-cells of the pia mater and the grey and white matter of the brain and cord. These authors stressed the many resemblances of the changes to those in anterior poliomyelitis in man.

Hurst (1931) also found that monkeys (*Macacus rhesus*) are susceptible to the virus of louping ill, a progressive ataxia of cerebellar type being the dominant clinical feature, while histologically a more or less diffuse encephalo-myelitis is accompanied by a massive destruction of the Purkinjë cells of the cerebellum. In the mouse, but not in the monkey, intracytoplasmic structures, probably of the nature of inclusion bodies, were found in the nerve-cells. Brownlee and Wilson (1932) could not demonstrate specific inclusion bodies in the nervous tissues of sheep suffering from louping ill, though intra-cellular bodies of a non-specific nature were found in the large nerve-cells of the spinal ganglia.

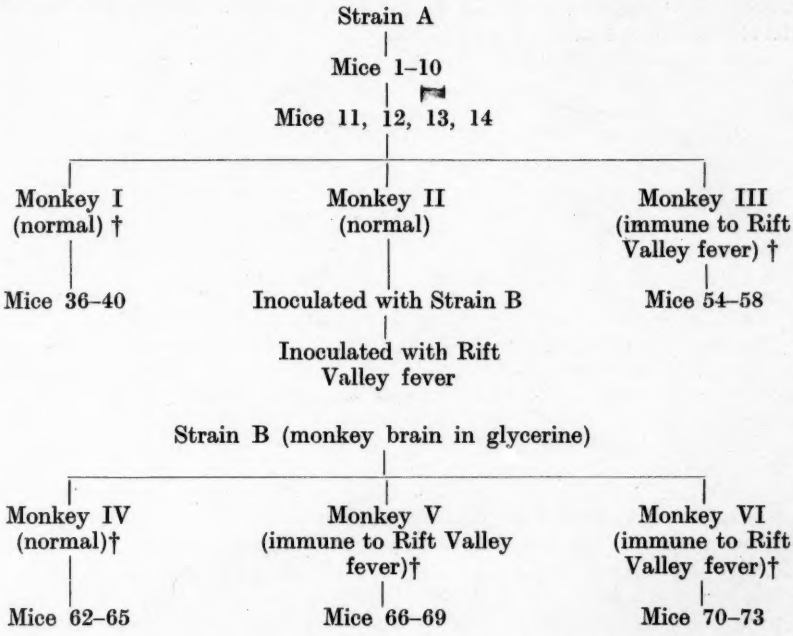
The fact that louping ill is transmissible to sheep, mice and monkeys suggested a similarity to Rift Valley fever, which is also pathogenic for the same species, although the virus of Rift Valley fever shows no neurotropic tendencies. It might, however, be possible for Rift Valley fever to protect against louping ill in the way that, according to Dinger and Snijders (1931), an attack of dengue fever protects monkeys against subsequent infection with yellow fever.

As Rift Valley fever produces a non-fatal disease in *rhesus* monkeys (Findlay, 1932), it was possible by using these animals to examine the question of cross-immunity between louping ill and Rift Valley fever.

THE TRANSMISSION OF LOUPING ILL TO MONKEYS (*MACACUS RHESUS*).

Two strains of louping ill virus were available; strain A, which was obtained through the kindness of Mr. W. S. Gordon, M.R.C.V.S., of the Moredun

Institute for Animal Diseases, Gilmerton, Edinburgh, consisted of virus in dried mouse brain ; while strain B, for which I am indebted to Dr. E. Weston Hurst, of the Lister Institute, consisted of virus in monkey brain preserved in glycerine.



Strain A, after two passages through mice, was inoculated intracerebrally into three monkeys (*Macacus rhesus*), each monkey receiving 1 c.c. of a 1 in 10 suspension in saline of infected mouse brain, the suspension having first been centrifuged for five minutes at 2000 revolutions per minute to throw down gross particles. Monkeys 1 and 2 were normal, but Monkey 3 had suffered from Rift Valley fever three months previously, and was immune to a second inoculation of Rift Valley fever virus given one week before the injection of the louping ill virus. All three monkeys developed symptoms, but while Monkeys 1 and 3 died from the infection, Monkey 2 eventually recovered, and was immune to a second inoculation of louping ill virus.

In Monkeys 1 and 3 the clinical symptoms were very similar to those described by Hurst (1931). Monkey 1 did not exhibit any significant rise in temperature, but in Monkey 3 the temperature rose to 103·2° F. on the fourth day, followed by a fall and a further rise on the sixth day after inoculation (Chart 1). Before death there was a rapid fall in temperature to a subnormal level. Monkey 1 was killed on the thirteenth day after inoculation when moribund, Monkey 3 on the eleventh day. In Monkey 2 there was also a saddle-back type of temperature (Chart 2), as well as in Monkey 4, inoculated

with Strain B virus (Chart 3). Monkey 4 was the only one of those inoculated with Strain B to show any definite rise in temperature. Monkeys 4 and 5 were killed when moribund on the eleventh day, Monkey 6 on the twelfth day after inoculation. Thus Monkeys 5 and 6, which had suffered from Rift Valley fever six and three months previously, died from louping ill after the same interval as normal monkeys.

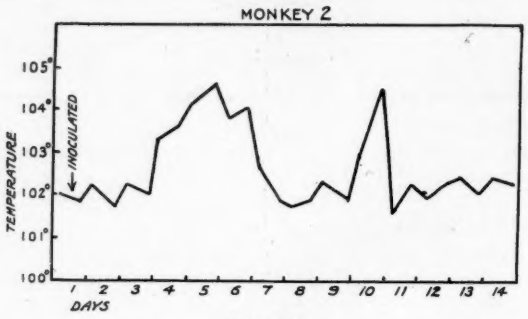


CHART 2.

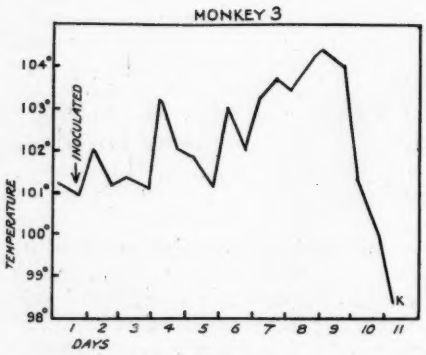


CHART 1.

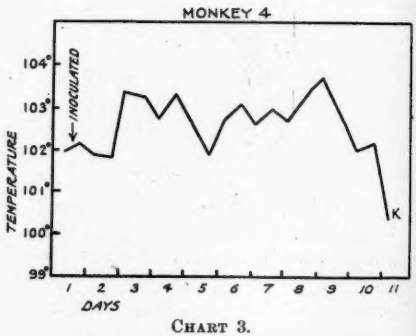


CHART 3.

Clinical symptoms were also similar in the two groups of monkeys, and were first noticeable on the fourth or fifth day after inoculation. The animals became less active and tended to mope on the floors of their cages. Muscular weakness gradually developed and there was loss of appetite. The weakness was most marked in the hind limbs, so that when the animals moved they were unsteady and tended to lose their balance. At the last they could scarcely move about their cages, though some power was retained in the upper limbs till shortly before death, as shown by the characteristic clinging with the arms to the sides of the cage. The deep reflexes were present throughout, and so far as could be judged there was no loss of sensation. Monkey 3 suffered from diarrhœa for the last three days of its illness, but in all the monkeys there was

no involvement of the sphincters. Monkeys 3, 4 and 6 exhibited a fine tremor of the head shortly before they were killed, and in these nystagmoid movements of the eyes were well seen. In Monkey 2 the symptoms were of some interest. Four days after inoculation it became quiet and sat hunched up, with its head sunk on its chest. Six days after inoculation it refused food, and for the next five days took very little nourishment while it was weak, failed to move about its cage and was obviously very ill. With the fall of temperature on the eleventh day after inoculation, however, there was a sudden improvement in the monkey's condition. It no longer looked dejected and began to eat with avidity, its former healthy condition being regained in the course of the following few days. Five weeks after the first injection, 1 c.c. of a 1 in 10 suspension in saline of the brain of Monkey 5 was inoculated intracerebrally into this monkey. There were no clinical symptoms, and the monkey remained in perfect health.

HISTOLOGICAL CHANGES.

In conformity with the clinical symptoms, which are those of a cerebellar ataxia, the most constant lesions were found in the cerebellar cortex, which was affected in all monkeys. Many of the granular cells in the cerebellar cortex were definitely pyknotic, while in Monkeys 3 and 4 there were petechial hæmorrhages in this region. The most striking lesion, however, was the extensive degeneration, and in many areas the almost total disappearance of the Purkinjë cells, with only here and there a recognizable survivor, without any proliferative changes in the neighbouring microglia cells. Of the few remaining Purkinjë cells scarcely any were normal, pyknosis, vacuolation and distortion being very common. In other parts of the brain and cord the lesions were of varying intensity, and in all cases were much less extensive than in mice. Meningeal infiltration, which was restricted to a few mononuclear cells, was always very slight. In Monkey 3 it was practically non-existent, most marked in Monkey 6. In this animal also the cerebral cortex showed the greatest degree of involvement. The lesions consisted of slight perivascular cuffing, microglial proliferation, an increase in the number of invading cells, and occasional degeneration of the neurones with neuronophagia. In all the other monkeys these changes were best seen in the mid-brain and pons. The infiltrating cells consisted for the most part of lymphocytes with a few plasma- and plasmacytoid cells, and only very occasionally a polymorphonuclear leucocyte. As noted by Hurst, in no case was the occipital cortex affected, and in only two was the cornu ammonis involved. Similar changes to those in the brain-stem were found irregularly and to a lesser extent in the cord. Acute necrosis of the anterior horn-cells was rarely seen, though in Nissl preparations there was a considerable degree of chromatolysis. As pointed out by Brownlee and Wilson (1932) in the case of mice and pigs, the nerve-cell damage in this disease is in no way proportionate to the interstitial changes.

CYTOPLASMIC INCLUSIONS.

Hurst (1931) described the presence in many of the nerve-cells of mice of structures which definitely fall into the class of virus inclusion bodies. Similar

inclusion bodies were not found in the nerve-cells of monkeys in the present investigations, as the small eosinophilic granules observed in many of the cells in the brain-stem and cord were equally present in the nerve-cells of normal monkeys. As, however, the brains of a number of mice with louping ill were available for examination, it was possible to confirm the presence of intra-cytoplasmic bodies in the neurones of mice. The appearance of these bodies is seen in Fig. 1. They are most numerous in the neurons of the pons and mid-brain, rather less frequent in the cord and Purkinjë cells and rather rare in the cerebral cortex, where they are always small. Unlike Negri bodies, there is no special concentration in the cornu ammonis. The bodies are restricted to the cytoplasm of nerve-cells, and have not been encountered in

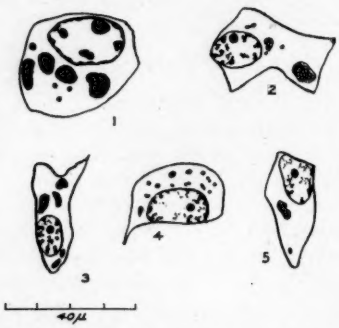


FIG. 1.—Cytoplasmic bodies in the nerve-cells of mice with louping ill.

1. Stained with iron-hæmatoxylin; partial decolorization with iron alum; fixed in sublimate formol.

2–5. Stages in the development of cytoplasmic bodies; stained with methylene-blue and eosin, fixed in sublimate formol.

neuroglia, vascular endothelium, plasma-cells, lymphocytes, or polymorpho-nuclear leucocytes. They are apparently destroyed by fixation in such mixtures as Zenker's fluid, and, as Hurst has described, are best seen after sublimate formol fixation followed by eosin methylene-blue staining. Even after 48 hours' staining in Giemsa they appear only faintly pink in colour, while prolonged staining with phloxine is also necessary to obtain definite coloration. The appearances following partial differentiation in iron alum after staining with Heidenhain's hæmatoxylin are shown in Fig. 1, 1, where the larger bodies are still black throughout, while the smaller show a central area surrounded by a lighter halo. Attempts to stain the bodies by the Feulgen technique were negative or gave only a very faint reaction, so that apparently they are not composed of chromatin.

The bodies vary from 1 to 10μ, and are usually somewhat oval in shape when small, kidney-shaped or elongated when larger. The size and number of the bodies found within the same cell show considerable variation. No definite internal structure was seen within the bodies. In addition to the

nerve-cells of the brain and cord, the presence of the bodies was confirmed in the nerve-cells of the ganglia lying between the adrenal and the cortex. In the adrenals of two mice examined there were also found in the cytoplasm of some of the cells of the medulla small eosinophilic bodies, resembling in certain respects those present in the nerve-cells.

No adequate explanation of the absence of these intra-cytoplasmic bodies in the nerve-cells of the monkey as distinct from the mouse can be given. The adrenal medulla of the monkey also failed to show their presence. Possibly there is some analogy to rabies, where fixed virus fails to give rise to Negri bodies, while street virus produces them. As previously mentioned, Brownlee and Wilson (1932) failed to determine the presence of specific inclusion bodies in the nerve-cells of the sheep. Further work can alone determine the relation of the bodies to the virus of louping ill.

THE PRESENCE OF VIRUS IN THE BLOOD.

In sheep, according to Poole, Brownlee and Wilson (1930), virus is generally found in the blood at some stage of the infection, but may be absent throughout the illness. Attempts to detect the presence of virus in the blood of Monkeys 1, 3 and 4 by the inoculation of 0·05 c.c. of heart blood into the brains of mice failed, however, and on reinoculating these mice intracerebrally with the brains of mice dying of louping ill, they all succumbed in from six to nine days with symptoms typical of the disease.

In the case of mice, on the other hand, it has been possible to show that louping-ill virus is present in the heart blood shortly before death.

Mice 37 and 38 were killed when moribund, and their heart bloods added to physiological saline to form a dilution of 1 in 10; 0·05 c.c. of the suspension was then inoculated intracerebrally into each of six mice. These mice all died in from six to eight days with symptoms and histological changes characteristic of louping ill. An emulsion of the brains of two of these mice filtered through a British Berkefeld filter transmitted the disease to other mice. A similar experiment was carried out with the heart-bloods of Mice 66 and 67. Six mice were again inoculated intracerebrally, but only four died with symptoms of louping ill. In a third experiment, carried out with the heart-blood of Mouse 72, the result was negative, and no mice died as the result of the intracerebral inoculation of the blood.

IMMUNIZATION OF MONKEYS AGAINST LOUPING ILL.

As previously mentioned, Monkey 2, inoculated with Strain A virus, recovered after a severe illness, and was subsequently immune to an intracerebral injection of strain B virus. In order to determine whether it was possible to immunize monkeys with Strain B against Strain A, two *rhesus* monkeys, 7 and 8, were injected intracerebrally with 0·2 c.c. of a 1 in 20 dilution in physiological saline of a suspension of the brain of Monkey 6, which had been preserved in 50 per cent. glycerine in saline for 7 days. There were no definite symptoms following this inoculation, though about the seventh day after the injection both monkeys became rather quiet and subdued. Three

weeks after the first injection the monkeys both received 1 c.c. intracerebrally of a 1 in 10 suspension in physiological saline of the brains of Mice 66, 67 and 68. No reaction occurred in the monkeys, though 4 mice injected intracerebrally with the same emulsion died in six, six, seven and eight days after injection, with typical symptoms of louping ill. Monkeys may thus be immunized against louping ill, while the two strains of virus employed in these studies are capable of producing cross-immunization.

CONCLUSIONS.

1. Louping ill can be transmitted to monkeys (*Macacus rhesus*) by intracerebral inoculation.

2. A progressive cerebellar ataxia results in monkeys, while histologically there is produced an encephalo-myelitis with special involvement of the Purkinjë cells of the cerebellum.

3. In association with the meningo-encephalo-myelitis produced by the virus of louping ill in mice, there are found cytoplasmic inclusions within the nerve-cells ; no such cytoplasmic bodies are found in the neurones of monkeys.

4. Intracerebral inoculation of sub-lethal doses of louping ill virus produces an immunity in monkeys. The two strains of virus employed gave a cross-immunity.

5. There is no cross-immunity between louping ill and Rift Valley fever.

6. In mice, but not in monkeys, the virus of louping ill was present in the heart-blood.

REFERENCES.

ALSTON, J. M., AND GIBSON, H. J.—(1931) *Brit. J. Exp. Path.*, **12**, 82.
BROWNLEE, A., AND WILSON, D. R.—(1932) *J. Comp. Path. Ther.*, **45**, 67.
DINGER, J. E., AND SNIJDERS, E. P.—(1931) *Arch. Schiffs. Trop. Hyg.*, **35**, 497.
FINDLAY, G. M.—(1932) *Trans. Roy. Soc. Trop. Med. Hyg.*, **25**, 229.
GREIG, J. R., BROWNLEE, A., WILSON, D. R., AND GORDON, W. S.—(1931) *Vet. Rec.*, **11**, 325.
HURST, E. W.— 1931) *J. Comp. Path. Ther.*, **44**, 231.
M'GOWAN, J. P., AND RETTIE, T.—(1913) *J. Path. Bact.*, **18**, 47.
POOL, W. A., BROWNLEE, A., AND WILSON, D. R.—(1930) *J. Comp. Path. Ther.*, **43**, 253.

STUDIES ON PROTEIN-FREE SUSPENSIONS OF VIRUSES. IV. THE ANTIGENIC PROPERTIES AND SEROLOGICAL REACTIONS OF PROTEIN-FREE SUSPENSIONS OF PHAGE.

I. J. KLIGLER AND L. OLITZKI.

From the Department of Hygiene and Bacteriology, Hebrew University, Jerusalem.

Received for publication April 27th, 1932.

THE importance of the problem of virus immunity and the uncertainty attached to the nature of the immune bodies and of virus-antibody union (Andrewes, 1931) suggested a study of these phenomena with purified virus. As a point of departure we have re-examined the behaviour of phage, because the reported phage-antiserum reactions correspond in many respects to those observed in viruses.

In spite of the vast literature on the nature and properties of the lytic principle, there is no certainty as yet concerning the antigenic character of phage and the mode of phage-antiphage union. In his admirable critical review Hadley (1928) concludes his analysis of the literature bearing on this aspect of the problem in the following words : " From the somewhat confusing array of the reports which have now been examined, what shall we conclude regarding the bacteriophage as an antigen ? I do not believe a final answer can be given at present." Further, he states : " It seems to me, however, that the conclusions on this matter can never have a secure support until methods are devised to obtain the bacteriophage in a relatively pure state . . . before it is used as an immunizing antigen." We have attempted, by means of a purified phage, to study both the nature of the phage as an antigen and the nature of the antigen-antibody reaction, and these experiments constitute the theme of this paper.

EXPERIMENTAL.

ANTIGENIC CHARACTER OF A COLIPHAGE.

In all experiments reported in this paper we used the same coli-phage as in the experiments previously reported (Kligler and Olitzki, 1931). In the preparation of the phage and in its purification by adsorption and elution, we also employed the technique previously reported (Kligler and Olitzki, 1931).

With the purified protein-free phage we proceeded to immunize rabbits. The immunization was effected by repeated injections of measured quantities of phage determined by counting, on an agar plate, the number of phage elements in a given volume of fluid. The injections were given intravenously or intraperitoneally at three- to five-day intervals, and after a given number of injections the serum was tested for neutralizing antibodies. Control rabbits were immunized with the original unpurified broth phage in the same manner, and the potency of the sera thus produced compared with those obtained with protein-free phage suspensions.

The results of the immunizations were uniformly positive and conclusive. Anti-phage serum could be produced regularly with ordinary broth phage as well as with the purified phage provided a sufficient quantity of antigen was injected. The antibody response is dependent on the amount of antigen injected, but surprisingly small amounts of phage suffice to elicit neutralizing antibody.

The following protocols are illustrative :

(*a*) *Rabbit 44 :* Immunized with pure phage.

Injected	6/4 i.v.	0·2 c.c. eluted phage	=	400,000 plaques.*
	12/4 i.p.	0·4 ,, ,, ,,	=	800,000 ,,
	14/4 ,,	0·8 ,, ,, ,,	=	1,600,000 ,,
Total .	.	1·4 c.c. eluted phage	=	2,800,000 plaques.

Bled 23/4. Titre 1 : 2 partially neutralizing.
1 : 5 negative.

(*b*) *Rabbit 83 :* Immunized with pure phage.

Injected	6/4 i.v.	0·2 c.c. phage eluate	=	3 millions.
	12/4 i.p.	0·4 ,, ,, ,,	=	6 ,,
	14/4 ,,	0·8 ,, ,, ,,	=	12 ,,
Total .	.	1·4 c.c. phage eluate	=	21 millions.

Bled 23/4. Titre 1 : 160 still active.
1 : 320 negative.

(*c*) Titration of antiserum produced with purified phage. Neutralization at 37° C. for 24 hours.

Serum dilution.	Phage dilution. 5^{-3}.	5^{-4}.
1 : 10 . .	6† .	1
1 : 50 . .	18 .	3
1 : 200 . .	160 .	14
Control . .	180 .	26

Rabbit 45 : Immunized with broth phage.

Date.	Volume injected.	Number of plaques.
6 .	1 c.c. i.v. .	2 millions.
9 .	1 ,, ,, .	2 ,,
13 .	2 ,, i.p. .	4 ,,
20 .	4 ,, ,, .	8 ,,
26 .	6 ,, ,, .	12 ,,
Total .	14 c.c .	28 millions.

Bled 7 days later.
Titre 1 : 160 still active.
1 : 320 negative.

* Refers to plaques or " colonies " on agar plates when seeded with a specific culture.
† In phage titrations throughout this paper the numbers represent the plaques on agar plates.

It is clear from these protocols that about 20 million phage elements elicit a satisfactory antibody response, and that when that amount is injected, the pure phage behaves in the same way as the broth phage. Both sera are equally antilytic, but they differ in that the one prepared with the eluate contains only phage antibody, while that prepared with the original broth contains also bacterial antibodies.

SEROLOGICAL REACTIONS.

With the antilytic sera so obtained we conducted a series of experiments with a view to elucidating the nature of the antibody and the mechanism of its neutralizing or antilytic activity. In every case cross tests were made with both types of antisera against purified and unpurified phage. In this way it was possible to cross-check the results, and to rule out masking effects or extraneous reactions. Moreover, our technique was designed to give quantitative data. In most of the previous work varying dilutions of sera were tested against a constant amount of phage and the mixtures seeded in broth. This procedure measured the action of the serum against gross phage units, but did not preclude the possibility of the presence of an excess of phage. Arnold and Weiss (1924), realizing that this procedure was unsatisfactory, spread the serum-phage mixtures on agar plates seeded with the specific sensitive culture and estimated the degree of deviation of phage activity; but they, too, worked with constant volumes of phage, and hence with mixtures which were not always neutral. In our experiments we used simultaneously varying dilutions of sera against varying dilutions of phage, the concentration of the latter being determined by the number of plaques or "colonies" on agar plates seeded with a constant amount of culture spread over the entire plate, together with a constant volume of the respective phage or serum-phage mixtures. This procedure enabled us to measure the relative anti-lytic activity of the serum against different quantities of phage and the influence of various factors, such as concentration of phage, time, temperature, heat and complement, on the antilytic reaction, much as one measures the lytic effect of sera on bacteria.

Influence of Temperature on the Antilytic Reaction.

The following protocols represent typical data on the influence of temperature on the velocity of reaction and neutralizing power of antilytic sera. Both kinds of sera (prepared with eluted and broth antigens respectively) were tested against both types of antigens. Each experiment was repeated several times—often as many as 5 or 6 times, always with the same results. All dilutions of the broth phage were made in broth and of eluted phage in saline. The serum and phage were mixed and incubated, and at stated intervals 0·1 c.c. of the mixture was streaked out on agar plates, together with a constant amount of a specific sensitive culture; after incubating the plates for 24 hours at 37° C. the plaques were counted.

EXPERIMENT 1.—*Neutralization of Phage at Various Temperatures and Various Time Intervals with Antiserum Prepared with a Broth Phage.*

Anti-serum dilution.	Phage dilution.		10° C. 24 hours.	10° C. 36 hours.	10° C. 48 hours.	20° C. 24 hours.	20° C. 36 hours.	20° C. 48 hours.	30° C. 24 hours.	30° C. 36 hours.	30° C. 48 hours.
1 : 2	Original	10^{-3}	250	240	250	220	250	240	120	63	90
1 : 4	,,	10^{-3}	280	120	120	250	240	160	240	120	120
1 : 2	,,	10^{-4}	120	38	68	70	56	53	0	8	16
1 : 4	,,	10^{-4}	126	45	43	120	160	67	20	28	8
1 : 2	Eluted	10^{-3}	28	3	8	52	44	7	35	1	2
1 : 4	,,	10^{-3}	34	9	0	27	28	0	24	4	8
1 : 2	,,	10^{-4}	0	0	0	7	7	0	4	0	0
1 : 4	,,	10^{-4}	4	4	0	3	6	2	2	1	0
Control : Broth phage		10^{-3}				260	280	240			
,, ,,		10^{-4}				70	90	84			
Pure phage		10^{-3}				60	45	46			
,, ,,		10^{-4}				12	18	13			

EXPERIMENT 2.—*Neutralization of Phage at Various Temperatures and after Various Periods with Antiphage Serum Prepared with Broth Phage.*

(Serum dilution 1 : 50.)

Time in hours.	10° C. Original phage diluted. 10^{-3}.	10° C. Original phage diluted. 10^{-4}.	10° C. Eluted phage diluted. 10^{-2}	10° C. Eluted phage diluted. 10^{-3}.	20° C. Original phage diluted. 10^{-3}.	20° C. Original phage diluted. 10^{-4}.	20° C. Eluted phage diluted. 10^{-2}.	20° C. Eluted phage diluted. 10^{-3}.	30° C. Original phage diluted. 10^{-3}.	30° C. Original phage diluted. 10^{-4}.	30° C. Eluted phage diluted. 10^{-2}.	30° C. Eluted phage diluted. 10^{-3}.
2	280	22	500	60	280	28	500	60	90	24	480	42
4	200	26	500	70	83	13	500	50	29	6	400	28
8	86	12	500	50	56	10	280	37	24	5	240	16
16	80	7	240	40	35	1	240	12	17	2	120	14
Control (without serum) :												
24	360	40	500	60	320	17	450	40	320	22	380	38

EXPERIMENT 3.—*Neutralization of Phage at Various Temperatures with Serum Prepared with Pure Phage.*

(Duration of contact 6 hours.)

Serum dilution.	10° C. Original phage diluted. 5^{-5}.	10° C. Original phage diluted. 5^{-6}.	10° C. Original phage diluted. 5^{-7}.	10° C. Eluted phage diluted. 5^{-4}.	10° C. Eluted phage diluted. 5^{-5}.	10° C. Eluted phage diluted. 5^{-6}.	30° C. Original phage diluted. 5^{-5}.	30° C. Original phage diluted. 5^{-6}.	30° C. Original phage diluted. 5^{-7}.	30° C. Eluted phage diluted. 5^{-4}.	30° C. Eluted phage diluted. 5^{-5}.	30° C. Eluted phage diluted. 5^{-6}.
1 : 20	360	80	28	460	120	32	240	56	4	160	32	2
1 : 40	360	120	45	360	160	29	280	32	8	240	28	8
Control (without serum)	400	160	36	360	160	24	400	120	17	360	130	16

The protocols cited, as well as the other experiments, demonstrate a number of points. In the first place it is clear that both types of sera possess neutralizing power against both purified and unpurified phage. It is also apparent that the rate and degree of neutralization depend on the concentration of phage as well as on the temperature and the concentration of antiserum.

In Experiment 1, for example, with the same concentration of serum (1 : 2), neutralization is noted at 10° C. in 36 and 48 hours with the 10^{-4} dilution of original phage, but not with the 10^{-3} dilution. In this experiment also the neutralization of eluted phage appears to be more complete than the original phage, but this effect is only apparent, being due rather to lesser number of elements in the same volume of eluted phage.

Experiment 2 is cited because it shows clearly that antiserum prepared with unpurified phage appears to act better on unpurified than on purified phage. This reaction is apparently due to the bacterial antibodies, and an adsorption of some of the phage on the precipitate produced by the precipitins. The antiserum produced with pure phage behaves in exactly the same manner towards both pure and impure phages (see Experiment 3).

In general, then, these experiments show that the velocity of the antigen-antibody reaction is slow, and that the degree of neutralization is dependent upon three variables—temperature, concentration of serum, and concentration of phage. With suitable dilutions of serum and phage partial neutralization can be noted at 30° C. even after relatively short intervals (2–6 hours).

Effect of Heat and Complement on the Antilytic Serum.

The suggestion has been advanced by various authors, notably Otto and Munter (1922) and Arnold and Weiss (1924), that the antiphage reaction is similar to that of toxin-antitoxin. This suggestion is based on the observations that the rate of neutralization is slow, that it follows the law of multiple proportion, that after neutralization dissociation occurs, and finally that no complement is needed for the reaction. The significant points seemed to us the *rôle* of complement and the reversibility of the union.

Effect of Complement.

The *rôle* of complement in the neutralization process seemed to require further investigation, and the quantitative technique employed by us appeared likely to give conclusive data on this point. The following protocols present the results of some of the experiments made to test the influence of complement on the course of the phage-antiphage reaction. The experiments were conducted under a variety of conditions, with pure and ordinary phage antisera, and the influence of complement was determined on heated as well as unheated antisera.

EXPERIMENT 1.—*Effect of Complement on the Titre of a Pure Phage Antiserum.*

A constant amount of phage was mixed with varying amounts of antiserum with and without complement (1 : 5), incubated 5 hours at 28° C., and plated.

Serum dilution.	Serum plus complement.	Serum without complement.
80	0	20
160	2	65
320	3	105
640	28	120
1280	54	120
2560	120	110
Control	140	120

EXPERIMENT 2.—*Effect of Complement on Unheated and Heated Antiphage Serum.*

Antiphage serum, as indicated, was mixed with phage and incubated 24 hours at 28° C. and plated. The time of heating was 15 minutes. The complement concentration was 1 : 10.

	Broth phage.							
	10^{-2}.				10^{-4}.			
Serum dilution.	Serum alone.	Serum plus complement.	Heated serum 65° C.	Heated serum 65° C. plus complement.	Serum.	Serum plus complement.	Heated serum 65° C.	Heated serum 65° C. plus complement.
1 : 10	11	60	33	13	0	0	40	5
1 : 20	34	47	280	30	1	3	40	1
1 : 40	40	30	240	120	2	8	120	8
1 : 80	55	45	280	120	9	6	75	11
1 : 160	31	34	400	140	8	4	100	20

	Pure phage.							
	10^{-2}.				10^{-4}.			
1 : 10	20	20	400	80	12	16	13	8
1 : 20	30	32	500	220	20	24	120	4
1 : 40	64	100	500	180	30	2	160	40
1 : 80	180	140	500	400	30	7	180	64
1 : 160	240	280	600	500	20	18	160	120

Broth phage control (no serum) 10^{-3} { 400, 380 } 10^{-4} { 80, 96 }

Pure phage control (no serum) 10^{-2} { 500, 500 } 10^{-3} { 120, 160 }

EXPERIMENT 3.—*Effect of Complement on Impure Phage Antiserum ; Neutralization of Phage by Heated and Unheated Phage Antiserum with and without Addition of Complement.*

Complement concentration (1 : 5); neutralization 4 hours at 28° C.; serum heated 15 minutes at temperatures given.

	Broth phage.									
	Serum unheated.		Serum heated.							
Serum dilution.	Serum alone.	Serum plus complement.	60° C.	60° C. plus complement.	65° C.	65° C. plus complement.	70° C.	70° C. plus complement.	75° C.	75° C. plus complement.
1 : 40	110	13	200	38	360	11	400	76	460	28
1 : 80	160	18	260	22	170	90	360	40	380	150
1 : 160	164	36	240	360	320	360	400	200	400	500

	Pure phage.									
1 : 40	120	20	70	15	120	16	160	25	50	42
1 : 80	110	70	280	30	140	74	200	60	120	30
1 : 160	180	73	200	110	150	130	260	120	200	260

Broth phage dose = 500 plaques.
Pure phage dose = 300 plaques.

EXPERIMENT 4.—*Experiment with Pure Phage Antiserum ; Effect of Complement.*

The procedure was the same as in the preceding experiment, except that the sera were heated 25 minutes, the neutralization lasted 6 hours and the complement concentration was 1 : 5.

	Unheated serum.			Heated serum.					
Serum dilution.	Serum.	Serum plus complement.	Serum plus inactivated complement.	60° C.	60° C. plus complement.	60° C. plus inactivated complement.	65° C.	65° C. plus complement.	65° C. plus inactivated complement.	
1 : 40	56	0	90	200	5	120	320	80	360	Pure phage
1 : 80	280	8	120	360	4	280	360	180	..	
1 : 160	360	30	240	320	46	320	360	320	..	
1 : 40	120	9	65	320	6	90	320	65	260	Broth phage
1 : 80	160	18	80	320	22	240	320	240	..	
1 : 160	320	36	280	360	80	320	320	280	..	

Controls.*	Normal serum.	Normal serum plus complement.	Normal serum plus inactivated complement.
Pure phage	360	320	320
Broth phage	280	320	300

The results obtained differ from those hitherto reported. In all our experiments complement proved an important factor in the reaction, providing the masking effect of an excess of phage was eliminated. Using a small amount of phage (about 150 plaques) and varying dilutions of serum, it was found that the neutralizing power was enhanced by complement so that the titre of the serum was increased 8-fold—from 1 : 160 to 1 : 1280. Similarly it was found that complement could to some extent reactivate serum inactivated by heating at various temperatures, provided the antibody had not been completely destroyed. The differences were particularly striking when the incubation period of the phage-antiphage mixtures was relatively short. In the type of experiment usually employed, with an incubation of 24 hours, the effect of complement is entirely masked, and this accounts perhaps for the negative results reported hitherto.

DISSOCIATION OF PHAGE-ANTIPHAGE COMPLEX.

Of importance also from the standpoint of the mechanism of the reaction are the findings of Otto and Munter (1922), and Weiss (1927). These authors claim that neutralized mixtures behave like toxin-antitoxin in that they can again be dissociated and active phage liberated. These findings correspond with those reported by Todd (1928), Andrewes (1928) and others with viruses. Muckenfuss (1928), who repeated the experiments of Otto and Munter and Weiss, was unable to confirm their observations.

* In the case of normal serum controls a 1 : 40 dilution was used.

We attempted to determine whether a neutral mixture of phage and antiphage serum could be dissociated either by dilution, as suggested by Otto and Munter, or by trypsin digestion of the antibody—the method employed by Weiss. We also employed the method of specific elution. All our experiments gave negative results when neutral mixtures—that is, mixtures which did not have an excess of phage—were used.

The accompanying protocols are typical of the results obtained :

EXPERIMENT 1.—*Dissociation of Phage-Antiphage Mixtures : Pure Phage Antiserum.*

A given amount of phage (500 [A] and 100 [B] plaques respectively) was mixed with antiserum (1 : 40), with and without complement, incubated 4 hours at 20° C. and 30° C. respectively, and then diluted as shown and plated. One set of plates was made immediately after the dilution, and the other after the dilution had stood for 2 hours at room temperature.

		First plating after incubation.						Second plating after 2 hours at 20° C.					
		1/1.	1/4.	1/8.	1/16.	1/32.	1/64.	1/1.	1/4.	1/8.	1/16.	1/32.	1/64.
Phage suspension A :													
20° C.	Phage plus antiserum	400	160	120	80	60	24	400	200	160	72	46	32
	Phage plus antiserum plus complement	220	120	80	28	16	3	240	120	90	25	9	6
	Phage plus normal serum	500	240	140	120	100	57	500	240	160	64	35	24
30° C.	Phage plus antiserum	260	120	80	40	18	8	280	180	80	64	43	24
	Phage plus antiserum plus complement	130	55	44	22	14	7	160	50	46	12	16	8
	Phage plus normal serum	500	320	160	140	80	60	500	240	160	80	56	32
Phage suspension B :													
20° C.	Phage plus antiserum	22	10	6	2	0	..	6	11	8	7	2	..
	Phage plus antiserum plus complement	0	0	0	0	0	..	0	0	0	0	0	..
	Phage plus normal serum	95	18	10	6	0	..	87	25	13	6	0	..
30° C.	Phage plus antiserum	28	3	1	0	0	..	11	14	6	0	0	..
	Phage plus antiserum plus complement	0	0	0	0	0	..	0	0	0	0	0	..
	Phage plus normal serum	76	12	5	1	0	..	82	18	6	2	0	..

In the first experiment the plating method reveals the fact that in so-called neutralized mixtures of phage-antiphage, which inhibit lysis in broth (*e.g.* the first tube containing undiluted serum phage mixture), there is an excess of phage present. The dilution of such a mixture decreases arithmetically both the phage and serum concentration, and consequently in tubes 2, 3, 4, etc., containing a concentration of serum no longer inhibitive, the presence of the phage, masked when the serum concentration was high, becomes apparent. However, mixtures which were completely neutralized and showed

EXPERIMENT 2.—*Dissociation of Phage-Antiphage Mixtures by Dilution and Trypsin.*

The experiment was arranged in the following manner:

I.		II.		III.		IV.	
Phage	0·2 c.c.	Phage	0·2 c.c.	Phage	0·2 c.c.	Phage	0·2 c.c.
Normal serum	0·2 ,,	Normal serum	0·2 ,,	Immune serum	0·2 ,,	Immune serum	0·2 ,,
Saline	0·1 ,,	Complement	0·1 ,,	Saline	0·1 ,,	Complement	0·1 ,,

(A) Every series was made with three dilutions of phage; after neutralizing 4 hours at 37° C., dilutions were made so that phage and serum were diluted 1 : 40, 1 : 80 and 1 : 160, and plated, with the following results:

Phage dilution.	I.				II.				III.				IV.			
	Undiluted mixture.	1:40.	1:80.	1:160.	Undiluted mixture.	1:40.	1:80.	1:160.	Undiluted mixture.	1:40.	1:80.	1:160.	Undiluted mixture.	1:40.	1:80.	1:160.
5^{-3}	800	120	44	23	800	100	60	26	640	80	10	6	600	80	16	4
5^{-4}	360	42	11	6	320	40	26	4	200	15	8	1	50	3	0	0
5^{-5}	76	12	7	2	120	14	3	1	50	8	0	0	28	2	0	0

(B) After the diluted mixtures had remained 2 hours at room temperature they were again plated:

5^{-3}	800	100	28	18	800	120	38	23	160	20	4	0	74	6	0	0
5^{-4}	320	35	16	10	320	36	18	11	38	4	0	0	30	3	0	0
5^{-5}	96	12	4	2	170	16	6	3	12	0	0	0	8	1	0	0

(C) At the time of the second plating the undiluted mixtures were divided into tubes, and to 0·1 c.c. portions were added (1) 0·50 c.c. Na_2CO_3 0·3%, (2) 0·05 c.c. trypsin, 0·1% solution in Na_2CO_3, incubated 20 hours at 37° C., and then plated, with the following results:

	Phage dilution.	I.	II.	III.	IV.
(1) Na_2CO_3 series	5^{-3}	800	800	52	22
	5^{-4}	300	360	8	0
	5^{-5}	100	130	4	0
(2) Trypsin series	5^{-3}	720	810	18	8
	5^{-4}	240	360	0	0
	5^{-5}	62	70	0	0

no excess of phage by the plating method did not "dissociate" on dilution and the growth in the broth was quite normal.

In like manner, as shown by protocol 2, the "dissociation" reported by Weiss by the method of trypsin digestion was clearly due to an excess of phage in the so-called neutral mixtures. Where the mixture was really neutral no dissociation occurred. In this respect our results confirm those reported by Muckenfuss (1928), who also failed to note any liberation of phage from neutral mixtures.

Distribution of Phage Antibody in Serum Fractions.

It has been shown by Arnold and Weiss (1924) that the antilytic substance is associated with the globulin fraction. We have been able to confirm this observation with sera produced with pure phage as well as with those obtained with ordinary phage. The following protocol is typical of the results obtained. The fractionation was carried out by 50 per cent. saturation with ammonium sulphate.

The Fractionation of an Antiphage Serum Produced with Pure Phage. Neutralization at 30° C.

	Original phage.					Eluted phage.				
	6 hours.			24 hours.		6 hours.			24 hours.	
	5^{-5}.	5^{-6}.	5^{-7}.	5^{-6}.	5^{-7}.	5^{-4}.	5^{-5}.	5^{-6}.	5^{-4}.	5^{-5}.	5^{-6}.
Whole serum :											
1 : 20	120	5	0	0	0	100	7	9	20	8	0
1 : 40	120	8	0	4	0	90	11	12	46	5	0
1 : 80	160	33	1	5	0	110	24	8	65	30	0
1 : 160	240	63	1	0	2	200	120	20	120	90	0
1 : 320	320	70	9	28	10	260	150	38	220	120	12
Globulin fraction :*											
1 : 20	80	6	4	0	0	80	28	4	76	6	3
1 : 40	160	28	11	5	0	100	45	8	120	10	2
1 : 80	120	52	14	26	8	120	73	3	150	65	18
1 : 160	180	45	12	32	19	160	60	24	120	87	33
1 : 320	280	97	28	63	14	320	120	21	320	160	28
Albumin fraction :											
1 : 40	240	83	29	84	21	360	180	36	360	120	32
1 : 80	240	94	22	105	23	320	160	36	360	160	36
Control :											
Phage without serum	280	86	28	90	29	360	160	32	360	140	54

* Redissolved in original volume and diluted as shown.

This protocol shows clearly that even by the crude method employed the bulk of the antilytic antibody is recovered in the globulin fraction of the serum. Furthermore, the globulin fraction reacts in the same manner as the whole serum, both as regards the velocity of neutralization, and the relation of concentration of phage and antibody to the rate and degree of neutralization.

DISCUSSION OF RESULTS.

The data presented help to clarify the existing confusion as to the nature of phage as an antigen and the behaviour of the specific antiserum produced by it. It appears that a desire to establish the virus or ferment nature of phage led to preconceptions as the manner in which it should react. Hence, while much valuable experimental data has been accumulated, there has often been a lack of a critical interpretation of the findings. This is clearly pointed out by Hadley (1928).

In the present stage of our knowledge it seems to us that any attempt to decide whether the bacteriophage is of the same nature as the viruses can be of little value. The fact is that both belong to that borderland where the territories of the living and non-living abut and the frontier is so ill-defined. That being the case we considered it more fruitful to confine ourselves for the time being to a study of the nature and reactions of phage and virus respectively, noting any similarities or differences in behaviour.

Viewed from this standpoint the results of our experiments are of interest in themselves, and at the same time offer a suggestive insight into the nature of the antigen-antibody reaction of entities of this order. The phage is clearly a definite antigenic entity. It elicits a specific antibody response in rabbits with little difficulty and with relatively small amounts of antigen. The reaction with its specific antibody is slow—much slower than one is accustomed to in the case of bacteria and their antibodies ; and for this, as well as other reasons, it has been assumed that the nature of the reaction is that of toxin and antitoxin. Our results suggest that this assumption is not fully justified. The accelerating effect of complement on the reaction, its influence in increasing the antilytic titre of the antiserum, and finally its power to reactivate partially inactivated antiserum, brings the phage-antiphage union into the class of antigen-amboceptor reactions (Belonovsky, 1927), such as bacteriolysis, etc.

This view is supported by the data showing that properly neutralized mixtures cannot be dissociated, or in other words, that the reaction is not reversible, as has been claimed by a number of workers. The slowness of the reaction may be easily explained by the size, dispersion and selective adsorption of the antigen—the very state that favours ready adsorption of the antigen by colloidal suspensions would retard the union with antibody, particularly if such union is considered as an electric phenomenon.

Whether these results can apply also to the serological reactions of viruses can only be determined by further investigation. A preliminary experiment with fowl-pox virus suggests that here also the addition of complement to the antiserum accelerates neutralization ; and though the results of one experiment with one virus do not warrant any generalization, it suggests that fowl-pox virus, like the coli-phage, produces an antibody which is not essentially different from one type of antibody produced by bacteria. In this connection it is of interest to note that Bedson (1928), in his studies on herpes virus, also concludes that " there does not appear to be any reason for supposing that viridical sera differ fundamentally in their mode of action from that of antibacterial serum."

CONCLUSIONS.

1. The pure phage is a definite antigenic entity.

2. The union of phage and antibody takes place slowly, but can be accelerated by a higher temperature and by the addition of complement. Complement can also reactivate partially inactivated phage antisera.

3. The reaction between phage and specific antibody would not appear to be reversible. Previously reported positive results appear to have been due to the presence of an excess of phage in the apparently neutral mixtures.

4. The phage antibody is associated with the globulin fraction of the serum.

5. A preliminary experiment suggests that in the case of fowl-pox virus also complement accelerates neutralization with an antiviral serum.

REFERENCES.

ANDREWES, C. H.—(1928) *J. Path. Bact.*, **31**, 671.—(1931) *Lancet*, **1**, 989.
ARNOLD L., AND WEISS, E.—(1924) *J. Infect. Dis.*, **35**, 603.
BEDSON, S. P.—(1928) *Brit. J. Exp. Path.*, **9**, 235.
BELONOVSKY, G. D.—(1927) *Z. ImmunForsch.*, **53**, 118.
HADLEY, PH.—(1928) *J. Infect. Dis.*, **42**, 263.
KLIGLER, I. J., AND OLITZKI, L.—(1931) *Brit. J. Exp. Path.*, **12**, 172.
MUCKENFUSS, R. S.—(1928) *J. Exp. Med.*, **48**, 709.
OTTO, R., AND MUNTER, H.—(1922) *Z. Hyg. InfektKr.*, **98**, 302.
TODD, C.—(1928) *Brit. J. Exp. Path.*, **9**, 244.
WEISS, E.—(1927) *J. Immunol.*, **13**, 301.

ANTIHÆMOLYSIN TITRES IN HÆMOLYTIC STREPTOCOCCAL INFECTIONS AND THEIR SIGNIFICANCE IN RHEUMATIC FEVER.

E. W. TODD, M.D.CAMB., D.P.H.DURH.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

Received for publication April 30th, 1932.

THE close relationship which exists between rheumatic fever and infection of the upper respiratory tract by hæmolytic streptococci was first pointed out by Coburn (1931); he indicated the need for immunological data as final proof of the ætiology of rheumatic fever. Collis (1931) and Glover and Griffith (1931) have also observed the association between hæmolytic streptococci and rheumatic fever. Wilson and Swift (1931), who approached the subject from a different angle, found that intravenous vaccination with hæmolytic streptococci diminished the incidence and the severity of rheumatic attacks. There is, therefore, considerable evidence that rheumatic fever is associated with infection by hæmolytic streptococci.

Todd (1932) described a method of titrating antistreptolysin in serum; and this technique supplies information from which a positive diagnosis of infection by hæmolytic streptococci can be made without a bacteriological examination.

The following paper gives the antistreptolysin titres of sera from cases of rheumatic fever, and it supplies immunological evidence that acute rheumatism is preceded by hæmolytic streptococcus infection.

Antistreptolysin titration was fully described in the earlier paper, and no further description of the technique is necessary, but a slight alteration has been made in the method of recording titres of sera. Formerly an antistreptolysin titre was recorded as the volume of serum required to neutralize $2\frac{1}{2}$ M.H.D. of hæmolysin; the titres were therefore always expressed as fractions of 1·0 c.c. This method of expression has now been modified, to facilitate comparison of different titres, by recording the reciprocals of the fractions, and describing the resulting whole numbers as " units " of antihæmolysin.

The different types of human hæmolytic streptococci, which are distinguishable from each other by precipitin and agglutinin reactions, produce serologically identical hæmolysin; and it is possible to diagnose response to infection by hæmolytic streptococci from observations on the titre of antihæmolysin in a patient's serum without reference to the type peculiarities of the infecting strain. Streptococcal hæmolysin is, however, species specific, being antigenically distinct from the hæmolysins of other species of bacteria, such as pneumococci and staphylococci. A high titre of antistreptolysin in a patient's serum is, therefore, evidence of infection by hæmolytic streptococci.

The following figures show: (A) That patients who are not affected by rheumatic symptoms usually have high antistreptolysin titres after infection by hæmolytic streptococci; (B) that acute rheumatic fever is accompanied by a high antistreptolysin titre.

A. THE RELATIONSHIP BETWEEN ANTISTREPTOLYSIN TITRE AND INFECTION BY HÆMOLYTIC STREPTOCOCCI.

The specificity of the reaction is shown in Tables I and II. Table I records low antihæmolysin titres in 6 normal horses, high antihæmolysin titres in 12 horses immunized with hæmolytic streptococci, and low titres in 17 control horses immunized with various bacterial antigens.

TABLE I.—*The Antistreptolysin Titre of Serum* (1) *from Normal Horses*, (2) *from Horses Immunized with Hæmolytic Streptococcus Toxin*, (3) *from Horses Immunized with Various Bacterial Antigens.*

1. Normal sera: 25, 33, 3, 5, 20, 33 units.
2. Hæmolytic streptococcus antisera: 3300, 5000, 1100, 1400, 1400, 250, 1400, 3300, 160, 1100, 1000, 1100 units.
3. Various antitoxic and antibacterial sera:
   Diphtheria, 5, 25, 33, 33 units.
   Tetanus, 17, 50, 25, 50 units.

Meningococcus, 3, 5, 50 units.
Staphylococcus, 50, 50 units.
Pneumococcus, 5, 3 units.
B. welchii, 11 units.
Non-hæmolytic streptococcus, 5 units.

Table II gives similar figures for human sera; 11 normal sera have titres ranging from 3 units to 100 units, sera from cases known to be infected with hæmolytic streptococci have high titres ranging from 140 units to 1700 units, while sera from diseases not associated with hæmolytic streptococci have titres ranging from 33 units to 110 units.

TABLE II.—*The Antihæmolysin Titre of Serum* (1) *from Normal Persons*, (2) *from Persons Infected with Hæmolytic Streptococci*, (3) *from Cases Infected with Other Organisms.*

1. Titres of normal human sera:
20, 20, 50, 10, 3, 100, 50, 50, 50, 33, 50 units.

2. Titres of sera from patients found by cultures to be infected with hæmolytic streptococci:

Scarlet fever cases (convalescent)—
(*a*) 330, (*b*) 200, (*c*) 330, (*d*) 200, (*e*) 330, (*f*) 500, (*g*) 330, (*h*) 200 units.

Erysipelas following mastoiditis—
(*i*) Acute stage . . . . . 1100 units.
Subsiding (eleven days later) . . . 1300 ,,

Tonsillitis—
(*j*) Subsiding . . . . . 170 units.
Convalescent (seventeen days later) . . 330 ,,
(*k*) Acute stage . . . . . 110 ,,
Convalescent (fifteen days later) . . 200 ,,
(*l*) Throat cultures negative up to 22.1.32, when hæmolytic streptococci appeared in almost pure culture.

22.1.32:	Onset of infection	33 units.
6.2.32:	Convalescent	100 ,,
10.2.32:	,, ...	110 ,,
17.2.32:	,, ...	140 ,,
23.2.32:	,, ...	140 ,,

Tonsillitis and sinusitis—
(*m*) Throat cultures negative up to 18.12.31, when hæmolytic streptococci appeared in pure culture.

19.12.31:	Onset of infection	100 units.
24.12.31:	Height of infection	1300 ,,
28.12.31:	,, ,, ...	1700 ,,
4. 1.32:	Convalescent	1000 ,,
11. 1.32:	,, ...	330 ,,
19. 2.32:	,, ...	170 ,,

Hæmolytic streptococcus pneumonia—

(*n*)	Subsiding	250 units.
	Convalescent (five days later)	330 ,,
	15 days after first titration	330 ,,
	21 ,, ,,	330 ,,

3. Titres of sera from diseases not associated with hæmolytic streptococci:

Osteomyelitis—

(*o*)	Discharging sinus for five years	33 units.
(*p*)	Hæmolytic staphylococcus infection	50 ,,
(*q*)	Sixteen months after onset	50 ,,

Bone and joint tuberculosis—

(*r*) 50, (*s*) 50, (*t*) 50, (*u*) 50, (*v*) 50, (*w*) 110 units.

Lobar pneumonia (pneumococcus)—

(*x*)	Convalescent	33 units.

Measles—

(*y*)	Convalescent	11 units.

Poliomyelitis—

(*z*)	Convalescent	14 units.

Table II shows that the antihæmolysin titre is frequently higher during convalescence than during the acute stage of infection. This is shown with greater emphasis in Table III, which gives the titres of nurses during their first year at the Presbyterian Hospital, New York, while under close clinical and bacteriological observation. The antihæmolysin titres were normal on entering the hospital, they remained normal during six months, when nurses were in contact with patients infected by hæmolytic streptococci, and they still remained normal during the acute stage of uncomplicated hæmolytic streptococcus pharyngitis. The antihæmolysin titres of nurses suffering from acute pharyngitis did not increase until convalescence was established, one to three months after infection.

TABLE III.—*The Influence of Hæmolytic Streptococcus Infection on the Antihæmolysin Titre of Student Nurses under Close Observation Clinically and Bacteriologically.*

I. Antistreptolysin titres on joining the staff of the Presbyterian Hospital, October, 1930 (no clinical or bacteriological signs of infection):
(*a*) 20, (*b*) 33, (*c*) 33, (*d*) 50, (*e*) 17, (*f*) 33 units.

II. Antistreptolysin titres of uninfected nurses before and after exposure to hæmolytic streptococcus cases (no clinical or bacteriological signs of infection).
October, 1930: (*g*) 50, (*h*) 50, (*i*) 50 units.
April, 1931: (*g*) 50, (*h*) 33, (*i*) 50 units.

III. Antistreptolysin titres during the acute stage of uncomplicated hæmolytic streptococcus pharyngitis.
(*k*) 50, (*l*) 33, (*m*) 25, (*n*) 50 units.

IV. Antistreptolysin titres after recovery from uncomplicated hæmolytic streptococcus pharyngitis (one to three months after infection).
(*n*) 140, (*o*) 200, (*p*) 200, (*q*) 330, (*r*) 140, (*s*) 200, (*t*) 140 units.
Note.—Serum from Nurse (*n*) was examined during acute pharyngitis, and also during convalescence six weeks later.

The low level of antihæmolysin which is frequently found during the acute stage of infection is also shown in Table IV, where the titres of cases of puerperal septicæmia and general peritonitis are recorded. Patients who died with positive blood-cultures had lower antihæmolysin titres than convalescent patients who had recovered from infection.

TABLE IV.—*The Antihæmolysin Titre of Serum from Cases of Puerperal Septicæmia and General Peritonitis Infected with Hæmolytic Streptococci.*

Septicæmia :—
Twelve fatal cases :
20, 5, 50, 33, 11, 100, 50, 25, 11, 125, 14, 30 units.
Three patients who recovered :
200, 330, 330 units.
Peritonitis :—
Three fatal cases :
25, 110, 17 units.
Two patients who recovered :
250, 500 units.
Daily observations on individual cases of septicæmia :

Case 1 :	29.12.31	14 units.
	30.12.31	14 ,,
	31.12.31	14 ,,
	1. 1.32	14 ,,
	4. 1.32 Died.	
Case 2 :	9. 3.32	17 units.
	15. 3.32	50 ,,
	17. 3.32 Died	50 ,,
Case 3 :	Cellulitis for three weeks followed by septicæmia.	
	29.12.31 Septicæmia	170 units.
	30–12.31 ,,	170 ,,
	1. 1.32 ,,	170 ,,
	4. 1.32 ,,	170 ,,
	26. 1.32 Recovered	330 ,,

Schlesinger (1930) and Sheldon (1931) observed that the naso-pharyngeal infection which precedes rheumatic fever is not immediately followed by rheumatic symptoms ; the infection at first appears to run a normal course, convalescence is established, and rheumatic symptoms then appear. The figures recorded above demonstrate the constancy of high antistreptolysin titres during convalescence from infection by hæmolytic streptococci at the time when rheumatic symptoms would be expected to appear. Titration of

the antihæmolysin in serum from acute rheumatic fever patients was, therefore, undertaken to investigate the relation between infection by hæmolytic streptococci and rheumatic fever.

B. THE CONSTANCY OF HIGH ANTISTREPTOLYSIN TITRES IN ACUTE RHEUMATIC FEVER.

Observations on antihæmolysin titres were at first confined to sera from patients at the Presbyterian Hospital, New York, who had been periodically examined for hæmolytic streptococcus infection of the upper respiratory

TABLE V.—*Antihæmolysin Titres of* (1) *Apparently Quiescent Rheumatic Subjects and* (2) *Patients with Acute Rheumatic Fever. Spring*, 1928, *to Autumn*, 1931.

(1) Apparently quiescent.		(2) Active rheumatic fever.	
Ell—	50 units.	Mai—	166 units.
Wyn—	50 ,,	She—	330 ,,
Sci—	50 ,,	Gai—	170 ,,
Bab—	33 ,,	Cli—	170 ,,
Doy—	50 ,,	Phi—	250 ,,
Tru—	110 ,,	O'Ha—	125 ,,
Han—	33 ,,	Fee—	330 ,,
Tol—	200 ,,	Gir—	500 ,,
Tro—	33 ,,	Ker—	1250 ,,
Jon—	100 ,,	Ste—	140 ,,
Wag—	50 ,,	Mur—	500 ,,
Mos—	50 ,,	Smi—	330 ,,
Zig —	20 ,,	Cro—	147 ,,
Rot—	33 ,,	Maz—	330 ,,
Mal—	50 ,,	Man—	500 ,,
Bar—	33 ,,	Wil—	170 ,,
Lac—	170 ,,	Rap—	170 ,,
Mak—	110 ,,	Bra—	330 ,,
Bus—	50 ,,	McC—	200 ,,
Cer—	50 ,,	Ric—	330 ,,
Tri—	50 ,,	Kon—	500 ,,
Coh—	100 ,,	Ros—	110 ,,
Cou—	33 ,,	Pro—	330 ,,
Hoe—	50 ,,		
Rom—	50 ,,		

tract ; subsequently sera were tested from cases of acute rheumatism in whom the bacterial flora of the upper respiratory tract was unknown. The following figures show that rheumatic patients whose symptoms are quiescent have usually, though not invariably, a low antihæmolysin titre ; on the other hand, patients with symptoms of active rheumatic disease have a high antihæmolysin titre. Similarly the antihæmolysin titre of individual patients rises during

an attack of acute rheumatism, falls during a period of quiescence, and rises again with recrudescence of rheumatic symptoms.

Table V gives the antihæmolysin titres of 25 apparently quiescent rheumatic subjects with mitral stenosis, but without evidence of active rheumatic disease. Throat cultures were taken twice a month from all the quiescent patients, and no evidence of infection by hæmolytic streptococci was obtained during the six months which preceded the serum tests. Four sera had abnormally high antihæmolysin titres, two sera had relatively high titres which are possibly within the normal range, while the remaining 19 sera had normal antihæmolysin titres. In contrast to these findings, 23 sera from patients with active rheumatic fever all gave abnormally high antihæmolysin readings.

In view of the occurrence of high antihæmolysin titres in a small proportion of quiescent patients, it seemed important to determine the titres of individual cases during activity and also during quiescence.

In Table VI, where these changes are recorded, the cases may be divided into three groups: Group I, Cases 1 to 6, had low antihæmolysin titres during quiescence and high titres during activity; Group II, Cases 7 to 11, had abnormally high titres during quiescence, and a further rise of titre occurred during periods of activity; Group III, Case 12, had pericarditis without polyarthritis or other rheumatic manifestations. She was the only case in which hæmolytic streptococci were not obtained from throat cultures, and also the only case in which a normal antihæmolysin titre was obtained during a rheumatic attack.

TABLE VI.—*The Changes in Antihæmolysin Titres in Rheumatic Subjects during Quiescence and during Recrudescence following Hæmolytic Streptococcus Pharyngitis.*

Group I:		
Case 1.—Gai—, girl, aged 14.		
20.12.30:	Quiescent	50 units.
5. 3.31:	Acute rheumatism	170 ,,
20.10.31:	Mild recrudescence	125 ,,
Case 2.—Cli—, boy, aged 16.		
4.10.30:	Quiescent	33 units.
4. 3.31:	Acute rheumatism	170 ,,
18.10.31:	Recovery	50 ,,
Case 3.—O'Ha—, woman, aged 22.		
24. 3.31:	Acute rheumatism	125 units.
6.11.31:	Recovery	50 ,,
13. 1.32:	,,	50 ,,
20. 3.32:	Acute rheumatism	250 ,,
Case 4.—Gir—, girl, aged 15.		
26. 3.31:	Acute rheumatism	500 units.
16. 5.31:	,, ,,	500 ,,
18.10.31:	Recovery	50 ,,
Case 5.—Phi—, man, aged 21.		
9. 3.31:	Acute rheumatism	250 units.
14.10.31:	Recovery	50 ,,

Case 6.—Han—.

10.12.30 :	Quiescent	50 units.
17. 3.32 :	Pharyngitis	50 ,,
31. 3.32 :	Acute rheumatism . . .	200 ,,
5. 4.32 :	,, ,, . . .	250 ,,

Group II :

Case 7.—She—, boy, aged 14.

2. 3.31 :	Acute rheumatism . . .	330 units.
7.11.31 :	Apparently quiescent . . .	200 ,,
24. 3.32 :	Acute rheumatism . . .	250 ,,

Case 8.—Ker—, girl, aged 14.

6. 1.31 :	Acute rheumatism . . .	1000 units.
28. 2.31 :	,, ,, . . .	500 ,,
20. 4.31 :	,, ,, . . .	500 ,,
18. 5.31 :	,, ,, . . .	1250 ,,
20.10.31 :	Apparently quiescent . . .	250 ,,
19.12.31 :	,, ,, . . .	200 ,,
20. 2.32 :	,, ,, . . .	140 ,,

Case 9.—Fee—.

16. 4.31 :	Acute rheumatism . . .	330 units.
18.10.31 :	Quiescent	170 ,,

Case 10.—Bra—.

18. 5.31 :	Acute rheumatism . . .	330 units.
12.12.31 :	Quiescent	200 ,,

Case 11.—Ric—.

7. 6.31 :	Quiescent	250 units.
28. 3.32 :	Acute rheumatism . . .	330 ,,
31. 3.32 :	,, ,, . . .	330 ,,
5. 4.32 :	,, ,, . . .	330 ,,

Group III :

Case 12.—Fag—, girl, aged 16.

18. 5.31 :	Acute pericarditis . . .	50 units.
18.10.31 :	Quiescent	50 ,,

In every case, with one exception, the antihæmolysin titre was higher during active rheumatic disease than during quiescence. Two theories may be suggested to explain why the titres in Group II did not return to normal during quiescence : (*a*) A quiescent case which has had an unusually high titre during activity, followed by a normally falling titre during convalescence, may give a high reading without signs of active rheumatic disease. Case 8 possibly falls into this class. She had three severe rheumatic attacks within eight months, and the highest antihæmolysin titre recorded in acute rheumatic fever was then attained ; after her symptoms had subsided the titre fell rapidly, but failed to reach a normal level within the nine months during which her serum was examined. (*b*) Continued unrecognized chronic infection may possibly keep the antihæmolysin titre at a relatively high level, without causing any clinical signs of acute rheumatism.

Table VII gives the results of repeated tests at short intervals on sera from cases of acute rheumatic fever.

TABLE VII.—*Repeated Antihæmolysin Determinations during Acute Rheumatism following Hæmolytic Streptococcus Throat Infections.*

(1) Man—, girl, aged 3 ; first attack.		
11.1.32 :	Acute rheumatism . . .	500 units.
14.1.32 :	,, ,, . . .	500 ,,
16.1.32 :	,, ,, . . .	500 ,,
18.1.32 :	,, ,, . . .	500 ,,
27.1.32 :	,, ,, . . .	500 ,,
28.1.32 :	Died.	
(2) Maz—, girl, aged 12 ; second attack.		
3.12.31 :	Acute rheumatism . . .	250 units.
24.12.31 :	,, ,, . . .	330 ,,
28.12.31 :	,, ,, . . .	330 ,,
4. 1.32 :	,, ,, . . .	330 ,,
11. 1.32 :	Condition improving . . .	330 ,,
19. 1.32 :	,, ,, . . .	330 ,,
23. 1.32 :	,, ,, . . .	330 ,,
27. 1.32 :	,, ,, . . .	250 ,,
3. 2.32 :	,, ,, . . .	250 ,,
9. 2.32 :	,, ,, . . .	250 ,,
18. 2.32 :	,, ,, . . .	200 ,,
21. 2.32 :	,, ,, . . .	200 ,,
4. 3.32 :	,, ,, . . .	200 ,,
(3) Wil—, man, aged 30 ; recrudescence.		
13.1.32 :	Acute rheumatic fever . . .	140 units.
15.1.32 :	,, ,, ,, . . .	140 ,,
18.1.32 :	,, ,, ,, . . .	170 ,,
29.1.32 :	,, ,, ,, . . .	140 ,,
3.2.32 :	Condition improving . . .	125 ,,
10.2.32 :	,, ,, . . .	110 ,,
14.2.32 :	Hæmolytic streptococcus pharyngitis	— ,,
16.2.32 :	,, ,, ,,	50 ,,
23.2.32 :	Rheumatic recrudescence . .	140 ,,

This table shows that the antihæmolysin titre is dependent on infection by hæmolytic streptococci, and that variations in the intensity of rheumatic disease, occurring after the initial pharyngitis has subsided, are not necessarily accompanied by corresponding variations in the antihæmolysin titre. Case 1 became progressively worse, and ultimately died, while the titre remained at a constant high level. In Case 2 the titre did not begin to fall until three weeks after clinical improvement had commenced. Case 3 contracted hæmolytic streptococcus pharyngitis while his antihæmolysin titre was falling during recovery from a previous attack of rheumatic fever. The onset of pharyngitis

was accompanied by a sharp fall in antihæmolysin titre, and the subsequent rise in titre coincided with a recrudescence of rheumatic symptoms.

Table VIII gives the antihæmolysin titres of three girls, belonging to one family, who all contracted sore throats infected with hæmolytic streptococci. One sister did not develop rheumatic symptoms, and her serum had a normal antihæmolysin titre one month after the onset of pharyngitis. The other two sisters had a sharp rise in antihæmolysin titre and they both developed rheumatic fever.

TABLE VIII.—*The Antihæmolysin Titres of Three Members of a Family who all Contracted Hæmolytic Streptococcal Pharyngitis in the following order: Mary, aged* 19, *non-rheumatic; Angelina, aged* 14, *Rheumatic* (*quiescent* 2 *years*); *Sadie, aged* 12, *Rheumatic* (*? quiescent* 6 *months*).

Date.	Mary.	Angelina.	Sadie.
9.1.32	P. — units.	W. 25 units.	W. 125 units.
10.1.32	P. — ,,	P. — ,,	W. — ,,
23.1.32	P.C. — ,,	P.C. — ,,	P. — ,,
6.2.32	P.C. — ,,	R. 50 ,,	R. 200 ,,
11.2.32	P.C. — ,,	R. 200 ,,	R. 250 ,,
13.2.32	P.C. 50 ,,	R. — ,,	R. — ,,
17.2.32	P.C. — ,,	R.C. 125 ,,	R. — ,,
21.2.32	P.C. — ,,	R.C. — ,,	R.C. 330 ,,

W. = Well before onset of pharyngitis. P. = Onset of hæmolytic streptococcus pharyngitis. P.C. = Convalescent from pharyngitis. R. = Rheumatic fever. R.C. = Convalescent from rheumatic fever. — = Serum not tested.

Table IX gives the antihæmolysin titres of 15 cases of acute rheumatic fever who had not been examined for bacterial infection. Comparison with

TABLE IX.—*Antihæmolysin Titre of Serum from Patients with Typical Rheumatic Fever whose Throats were not Previously Examined for Hæmolytic Streptococcus Infection.*

Name.	Date.	Units of antihæmolysin.
Eli—, first attack	July, 1928	250 units.
,, second attack	Aug., ,,	330 ,,
Smy—	July, ,,	330 ,,
Lin—	May, ,,	110 ,,
Con—	,, ,,	500 ,,
Moy—	April, ,,	330 ,,
Cha—	June, ,,	330 ,,
Pis—	May, ,,	330 ,,
Cos—	June, ,,	170 ,,
Bla—	,, ,,	125 ,,
Cro—	,, ,,	170 ,,
Lal—	July, ,,	250 ,,
Rom—	Feb., 1929	200 ,,
Fee—	April, 1931	330 ,,
Kou—	Nov., 1931	500 ,,
Ait—*	Dec., 1928	50 ,,

* Bronchitis, rheumatic heart disease with failure of compensation; no acute manifestations of rheumatism except pyrexia, which was possibly due to bronchitis.

Table V will show that these titres are indistinguishable from the titres of rheumatic fever patients known to have been infected with hæmolytic streptococci. The only normal titre occurred in a case with a doubtful diagnosis of active rheumatic fever.

Table X gives the antihæmolysin titres of 10 quiescent rheumatic subjects before and after typhoid inoculation. The general reaction, which followed the injection of typhoid vaccine, did not cause an increase of antihæmolysin in any of the sera. One case had an unusually rapid fall in antihæmolysin titre during the week following typhoid inoculation.

TABLE X.—*Antihæmolysin Titres in* 10 *Quiescent Rheumatic Subjects Before and After Typhoid-Paratyphoid Inoculation;* 10–14-*year-old Girls with Mitral Stenosis.*

Name.	Before inoculation.	7 days after inoculation.	21 days after inoculation.
Mon—	250 units	50 units	33 units
Bra—	110 ,,	110 ,,	50 ,,
Ray—	125 ,,	125 ,,	100 ,,
Pot—	50 ,,	50 ,,	50 ,,
Gam—	50 ,,	50 ,,	— ,,
Har—	100 ,,	100 ,,	— ,,
Kri—	170 ,,	170 ,,	— ,,
Mey—	110 ,,	110 ,,	— ,,
Cut—	50 ,,	50 ,,	— ,,
Byr—	50 ,,	50 ,,	— ,,

THE *RÔLE* OF HÆMOLYSIN IN RHEUMATIC FEVER.

The observations recorded in this paper naturally lead to speculation on the *rôle* of hæmolysin in rheumatic fever. McLeod (1929) has emphasized the toxic qualities of streptococcal hæmolysin, and it is not improbable that at least some of the symptoms of rheumatic fever may be caused by hæmolytic toxin absorbed from a local area of infection. The present work does not support either the allergic theory or the rival theory of direct intoxication. It is possible that streptolysin has a toxic action responsible for symptoms of rheumatic fever, and that this intoxication is combated by the formation of antihæmolysin; on the other hand, it is equally possible that hæmolysin plays no part in the rheumatic syndrome, and that antihæmolysin in the patient's blood is a sequel to infection by hæmolytic streptococci which has no antitoxic effect in rheumatic fever. The latter theory is supported by the observation that rheumatic symptoms do not usually appear until a high antihæmolysin titre is attained.

CONCLUSIONS.

1. A consistent increase of antistreptolysin occurs in the blood after infection by hæmolytic streptococci. This increased titre is found in simple uncomplicated infections, and also in infections, followed by rheumatic fever.

2. Equally high antistreptolysin titres occur in rheumatic fever patients who have been shown by cultures to be infected by hæmolytic streptococci, and in rheumatic fever patients who have not been examined bacteriologically.

3. This supplies immunological evidence that rheumatic fever is preceded by hæmolytic streptococcal infection.

I am greatly indebted to Dr. Alvin F. Coburn, of the Presbyterian Hospital, New York, for allowing me to test a large number of sera from his patients, for clinical and bacteriological details of the cases, and for many suggestions. I am also indebted to Dr. L. Colebrook, Dr. N. Gray Hill, Dr. L. J. M. Laurent and Dr. Ruby Inkster for samples of serum.

REFERENCES.

COBURN, A. F.—(1931) 'The Factor of Infection in the Rheumatic State,' Baltimore.
COLLIS, W. R. F.—(1931) *Lancet*, **1**, 1341.
GLOVER, J. A., AND GRIFFITH, F.—(1931) *Brit. Med. J.*, **2**, 521.
MCLEOD, J. W.—(1929) 'System of Bacteriology,' **2**, 59 (H.M. Stationery Office).
SCHLESINGER, B. E.—(1930) *Arch. Dis. Child.*, **5**, 411.
SHELDON, W.—(1931) *Lancet*, **1**, 1337.
TODD, E. W.—(1932) *J. Exp. Med.*, **55**, 267.
WILSON, M. G., AND SWIFT, H. F.—(1931) *Amer. J. Dis. Child.*, **42**, 42.

THE NATURE OF THE VACCINIA FLOCCULATION REACTION, AND OBSERVATIONS ON THE ELEMENTARY BODIES OF VACCINIA.

JAMES CRAIGIE, M.B., CH.B., PH.D., D.P.H.ST.AND.,

Research Associate.

From the Connaught Laboratories, University of Toronto, Canada.

Received for publication May 5th, 1932.

IF a suspension of vaccine lymph be brought into contact with vaccinia-immune serum specific flocculation will take place, provided that the test is carried out under suitable conditions. The demonstration of this reaction by Tanaka (1902) and by Freyer (1904) was followed by a number of conflicting reports, which have been reviewed by Gordon (1925) and by Schultz, Bullock and Lawrence (1928). Gordon not only definitely established the existence and specificity of the reaction, but also demonstrated that vaccinia "agglutinating" serum specifically flocculated extracts of smallpox crusts. The sera were prepared by repeatedly injecting rabbits with suspensions of lapine (*i. e.* rabbit vaccinia). Schultz and his co-workers, on the other hand, employed

an unsuitable technique and failed to elicit the reaction. Their negative findings and the conflicting reports in the literature led them to suggest that the results of Gordon and of his predecessors were referable to a reaction between secondary infectors in the vaccine suspensions and antibodies to these contained in the immune serum.

Gordon's work was confirmed by Burgess, Craigie and Tulloch (1929), who applied his " agglutination " technique to crusts obtained from cases of variola minor and of varicella and showed that the test was of diagnostic value. These authors employed the term " flocculation reaction," since the nature of the reaction (agglutination or precipitation) was not known. Havens and Mayfield (1931) confirmed that the sera of man or rabbits acquire specific flocculating qualities for vaccinia suspensions after vaccination. They also applied the reaction to smallpox and chickenpox crusts, but their results, with the technique employed, were rather discouraging. In a more recent paper, however, these authors (1932) confirm the diagnostic value of the flocculation test. A further study of the reaction was made by Craigie and Tulloch (1931), who demonstrated that the reaction could be elicited with extracts prepared from tissues injected with strains of vaccinia free from secondary infectors and that the reaction was specific for vaccinia virus irrespective of organ or animal species. Methods of freeing the active material from substances which inhibited or obscured the reaction were developed and the results obtained with " antigen " purified to a considerable extent suggested that the reaction was, in nature, a precipitin reaction. Thompson and Buchbinder (1931), on the other hand, using suspensions freed only from grosser particles, consider that the reaction is an agglutination reaction.

With a suitable technique, variola and vaccinia flocculations may be obtained which are as clear cut and as obvious as any bacterial agglutination. It would seem desirable, therefore, to indicate briefly the main factors which tend to obscure or inhibit the reaction and which doubtless have been responsible for failures to elicit it. The factors fall into three groups: (1) Adventitious material—tissue particles and protein and ether-soluble substances—in the antigen suspension. (2) Lability of the specific " antigen " under certain circumstances at 56° C. (3) An inhibitory factor, present in rabbit serum, and which may come into play under certain conditions when the serum has a low content of flocculating antibody. Observations on this inhibitory factor will be published shortly; the other factors and methods whereby they may be largely eliminated have already been discussed (Craigie and Tulloch, 1931).

THE INFLUENCE OF SEITZ FILTRATION ON VACCINIA EXTRACTS.

When extracts of lapine are passed through a Seitz EK filter the virus is retained, while a variable amount of the flocculable substance passes through (Tulloch, 1932). A certain amount of this substance is, however, adsorbed by the material composing the filter disc, but this adsorption may be prevented by previously filtering diluted normal rabbit serum through the disc. Without serum treatment of the disc the amount of flocculable substance which passes the filter is very variable, depending upon the amount of flocculable substance

in the extract, the volume filtered, the character of the particular batch of lapine and its previous treatment. Under suitable conditions filtrates fully as active in the flocculation reaction as the original extract may be obtained, and frequently, in its stronger concentrations, the filtrate may give better flocculation than the extract. An example of this, which serves to emphasize how flocculation may be inhibited in plain extracts, is given in Table I.

The extract was filtered through a serum-treated disc at 10 cm. negative pressure. The first 3 c.c. of filtrate, consisting of saline retained by the disc from its previous washing, were discarded, and the filtrate collected in fractions thereafter. It will be noted that the unfiltered extract developed a specific opacity, but that sedimentation was interfered with.

TABLE I.—*Extract of Lapine V 85 = moist pulp 1 in 50.*

	Antivaccinia serum 1 in 20. Dilutions of extract = moist pulp diluted—					Normal rabbit serum 1 in 20. Dilutions of extract = moist pulp diluted—				
	1 in 400.	1 in 800.	1 in 1600.	1 in 3200.	1 in 6400.	1 in 400.	1 in 800.	1 in 1600.	1 in 3200.	1 in 6400.
Unfiltered extract	000 tr.	00+	00 tr.	0 tr.	—	0—	—	—	—	—
First 6 c.c. of filtrate	+++	+++	+	tr.	—	—	—	—	—	—
Second 6 c.c. of filtrate	+++	+++	++	+	—	—	—	—	—	—

1 hour at room temperature, then 16 hours at 55° C.
000, etc. = degrees of opacity.
+++, etc. = degrees of precipitation.
tr. = trace.

The Seitz EK filter, although it permits the passage of the flocculable substance, retains vaccine virus, since filtrates produce no observable lesion on the skin of the rabbit. The flocculable substance is therefore not the virus, and the flocculation reaction is essentially a precipitin reaction.

THE ELEMENTARY BODIES OF VACCINIA.

In the case of some filterable viruses minute bodies have been demonstrated in the lesions produced by the virus. In the past there has been a tendency to dismiss these elementary bodies either as artefacts or as non-specific particles derived from affected cells. Recently, however, in the cases of fowlpox (Woodruff and Goodpasture, 1929; 1930) and psittacosis (Bedson, 1932), evidence has been brought forward that these bodies are indeed the virus.* The latter author has shown that in psittacosis the elementary bodies, after being washed twice by centrifugation, retain the skin-virulence of the original suspension and are specifically agglutinated by immune serum.

The elementary bodies of vaccinia and variola were first described by Paschen (1906). Paschen observed that they were specifically agglutinated by immune serum (1913; 1924). Recently Nauck and Paschen (1932) have shown that the Paschen bodies multiply in tissue cultures of vaccine virus (up to the 35th serial culture), and that their number in a culture is related to

* At the time when this paper was dispatched for publication the author had not access to the paper by Eagles and Ledingham (1932), showing that the virulence of Berkefeld filtrates of vaccinia virus resided in the Paschen bodies.—ED.

virulence. Ledingham (1931) has elaborated a method of obtaining suspensions of the elementary bodies of vaccinia and of fowlpox with which agglutination (hanging-drop) tests could be carried out. Briefly, the method consists in grinding material harvested from early lesions with distilled water, extracting with ether, centrifuging down the elementary bodies at high speed, resuspending them in formol saline and removing the grosser particles from the suspension by centrifugation. With suspensions prepared in this way Ledingham (1932) showed specific agglutination in high dilutions of appropriate immune serum.

INVESTIGATION OF THE NATURE OF THE VACCINIA FLOCCULATION REACTION.

Since it has been demonstrated that the Seitz-filterable flocculable substance which is specific to vaccinial material is not the virus, the question of its relationship to the virus arises. Also, the recent work of Ledingham on the agglutination of the elementary bodies of vaccinia raises the question of their relationship to flocculable substance. Both questions have been investigated together in view of the ætiological significance which has been attached to the elementary body.

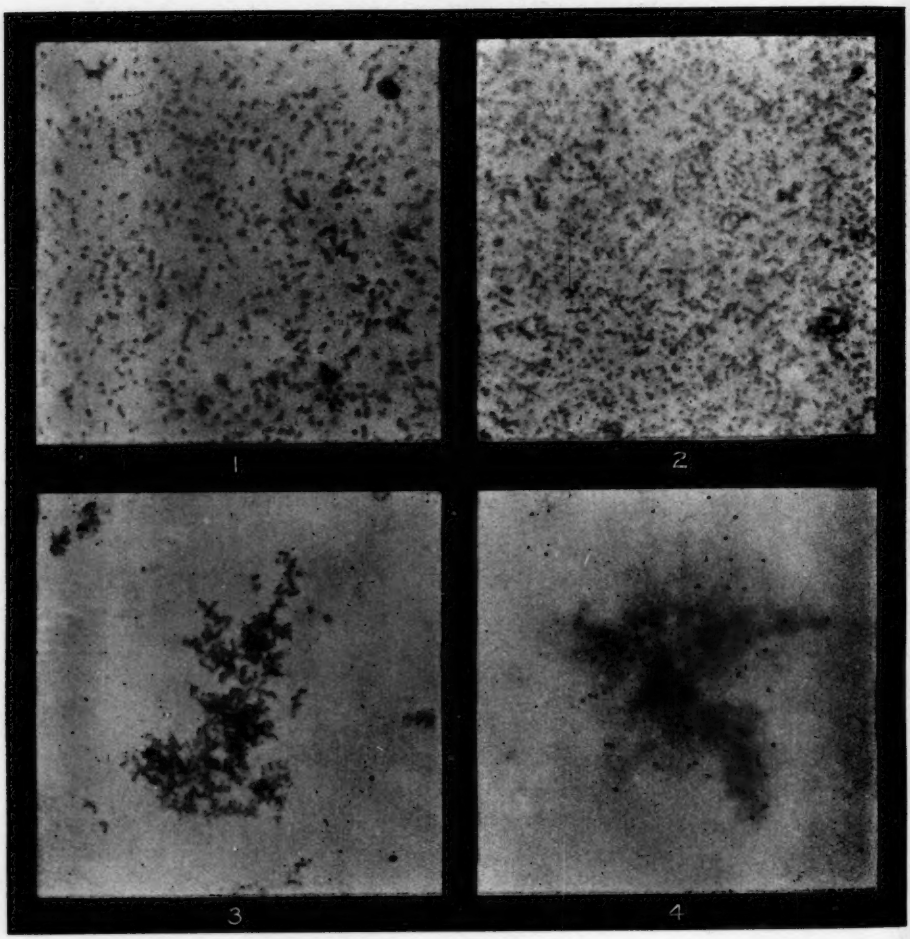
The Preparation of Elementary Body Suspensions.

Trial was first made of Ledingham's method of preparing suspensions, but the yield obtained was insufficient for the projected investigation. In addition convincing evidence was not forthcoming that the flocculation of the suspension was due primarily to agglutination of elementary bodies and not to traces of flocculable substance carried over from the original material. Possibly the method was not given a sufficiently extensive trial, for a somewhat similar method was found to give a satisfactory yield of elementary bodies and was adopted instead.

This method was suggested by observations made in following out the appearance and increase of elementary bodies which occurs after vaccination. It was found that the best preparations were obtained as follows: Third-day lesions on the skin of a rabbit are lightly cleansed with distilled water and a drop of distilled water placed on the cleansed area. By gently scraping the underlying skin with a scalpel a suspension is obtained from which very thin films are spread on microscope slides after the manner of a blood-film. The films are stained after the method of Gasagrandi (1927). When dry they are fixed in absolute methyl alcohol (15 minutes), dried and stained in 1 in 20 Giemsa* for 20 hours at 55° C. After staining the slides are rinsed in tap- and then distilled water. Excess moisture is blotted off, and the preparations are immediately, before they have dried completely, differentiated in two changes of absolute alcohol (5 seconds each) and immediately rinsed in xylol.

Since preparations made in this way from third-day lesions show enormous numbers of elementary bodies lying free from cells and a remarkable freedom from minor particles (Fig. 1), the method by which the material was collected was applied on a large scale.

* Some samples of Giemsa will not stain elementary bodies. It is preferable to prepare a stock solution from Grubler's dry stain, thus: dry stain 0·30 gm., glycerine 12·5 c.c., methyl alcohol 37·5 c.c.


1
2
3
4

The back and sides of rabbits were lightly inoculated with diluted lapine* with the minimum of trauma. Three days after vaccination (in some cases 2 or 4) the animals were exsanguinated, and the skin, which showed a confluent reaction, pinned out on a level board. The skin was first lightly cleansed with distilled water. The lapine was then harvested with a scalpel directly into two successive 10-c.c. volumes of buffered distilled water (0·004 m. citric acid—Na_2HPO_4 buffer; pH 7·0) placed on the skin. The suspension so obtained was centrifuged for 5 minutes at 1500 r.p.m. and the supernatant collected. The deposit was shaken with a further 10 c.c. of buffered distilled water and again centrifuged. The two supernatants were pooled, and this constituted the crude elementary body suspension. It will be noted that grinding and ether treatment were avoided. It has been observed that under certain conditions ether treatment, apart from its possible influence on the virus, may reduce the yield of elementary bodies.

The crude elementary body suspensions were next centrifuged in an angle centrifuge (Bolaget) in flat tubes (4 to 5 mm. internal diameter) for 50 to 60 minutes at 3500 r.p.m. The supernatant was decanted and the tubes inverted on filter-paper to drain, after which the deposits were re-suspended in buffered distilled water. Centrifugation and removal of the supernatant was repeated. In each instance the deposit was taken up in a volume equivalent to that of the amount of crude suspension centrifuged. After the final centrifugation the deposit was thoroughly dispersed in buffered distilled water, the volume of this being equivalent to the volume of crude elementary body suspension from which the deposit was obtained. Finally the suspension of washed elementary bodies was centrifuged in an ordinary centrifuge in order to sediment everything but elementary bodies. Portions of the suspensions required for serological tests were formolized to 0·25 per cent. Washed suspensions of elementary bodies obtained in this way show, microscopically, a remarkable purity (Fig. 2). They are very opalescent, but their optical character is such that it is difficult to compare them with opacity standards. Indeed, in appearance, they resemble concentrated flagellar suspensions rather than bacterial suspensions.

The Agglutinability and Virus Content of Elementary Body Suspensions.

Elementary body suspensions prepared in the way described and the various supernatants obtained in the course of their preparation were examined in three ways: (*a*) By the flocculation test, (*b*) by inoculation on the skin of rabbits, and (*c*) microscopically. In addition, a portion of the first supernatant was filtered through a Seitz EK filter and subjected to the flocculation test.

* The lapine was derived from vaccine lymph (Connaught Laboratories), and had been passaged exclusively on the skin of rabbits.

DESCRIPTION OF FIGURES.

FIG. 1.—Elementary bodies from dermal lesion of rabbit three days after vaccination. Two staphylococci are included in field. Giemsa, × 2000.

FIG. 2.—Elementary body suspension, V 140. Washed. Giemsa, × 2000.

FIG. 3.—Elementary body suspension, V 140. Action of antivaccinia serum. Giemsa, × 2000.

FIG. 4.—First supernatant, V 140. Action of antivaccinia serum. Giemsa, × 2000.

TABLE II. *Lapine V*

Flocc

| | Antivaccinia serum §74. 1 in 20. Dilutions of fractions. | | |

ABLE I ine V 145.

Normal rabbit serum. 1 in 20. Dilutions of fractions.							Test for virus content.					
1 in 50.	1 in 100.	1 in 200.	1 in 400.	1 in 800.	1 in 1600.	1 in 3200.	10^3.	10^4.	10^5.	10^6.	10^7.	10^8.
n.t.	−	−	−	−	−	−	++++	++++	++++	++	+	−
n.t.	−	−	−	−	−	−	+++	++	−	n.t.	n.t.	n.t.
n.t.	−	−	−	−	−	−	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
−	−	−	−	−	n.t.	n.t.	++	+	−	n.t.	n.t.	n.t.
−	−	−	−	−	−	n.t.	++++	++++	++++	++	+	−

++++ = confluent take.
+++ = semi-confluent.
++ = scattered papules.
+ = one or two papules.

However, since the "filterable antigen" gives a demonstrable precipitate in dilutions higher than that in which agglutination of elementary bodies from the same amount of pulp can be detected, the reaction is predominantly a precipitin reaction. The most striking feature of these experiments is the finding that vaccinia virus can be repeatedly washed in the centrifuge and that the washed suspension of virus, which possessed a degree of infectivity similar to that of the crude extract from which it was obtained, consists, apparently solely, of enormous numbers of elementary bodies. This association is strong evidence in favour of the view that the elementary bodies are the virus. In fact, since these structures show such a striking regularity of size and shape, increase so enormously in numbers during the early development of the vaccinial lesion and maintain such a close relationship to infectivity through the processes of fractional and repeated centrifugation, it would be remarkable if they were not vaccinia virus.

The Relationship between Flocculable Substance and Elementary Bodies.

In order to determine whether the Seitz-filterable flocculable substance and the elementary bodies of vaccinia are related, cross-absorption tests have been carried out. In performing such tests care is necessary in adjusting the relative proportion of antigen and antiserum since, in the case of the filtrate, excess of antigen cannot be removed from the mixture. A preliminary test in which various proportions of filtrate and serum are allowed to stand for 30 to 60 minutes at room temperature is useful in determining the probable optimum proportions. This was employed in the tests under consideration, and from the information derived two or more slightly different mixtures were set up.

In the tests one or other of two large batches of pooled antivaccinia serum were employed. These had a titre respectively of 1 in 160 and 1 in 320 for the filtrates and a rather higher titre for the elementary body suspensions. In

TABLE III.

Reference No. of batch of lapine.	Day after vaccination on which harvested.	Flocculation test. Highest dilution giving macroscopically visible precipitation.						Microscopical examination.		Virus content. Highest dilution giving confluent lesion.			
		C.	S_1.	F.S_1.	S_2.	S_3.	E.B.	S_1.	E.B.	C.	S_1.	S_2.	E.B.
V 138	3rd	1/1280	1/1280	1/800	1/80	n.o.	1/200	E.B.'s present	Apparently pure suspension of E.B.'s	$1/10^5$	$1/10^2$	n.t.	$1/10^5$
V 140	3rd	1/1600	1/1600	1/800	1/100	n.o.	1/400	Do.	Do.	$1/10^5$	$1/10^3$	$1/10^2$	$1/10^5$
V 143	3rd	1/1600	1/1600	1/1600	1/25	<1/12·5	1/200	,,	,,	$1/10^4$	n.t.	n.t.	$1/10^4$
V 145	3rd	1/1600	1/1600	1/1600	1/50	n.o.	1/400	,,	,,	$1/10^5$	$1/10^3$	$<1/10^3$	$1/10^{5*}$
V 149	2nd	1/400	1/400	n.o.	1/25	n.o.	1/50	,,	,,	$1/10^4$	$<1/10^3$	$<1/10^3$	$1/10^4$
V 151	4th	1/1600	1/1600	1/1600	1/50	<1/25	1/400	,,	,,	$1/10^5$	$<1/10^3$	n.t.	$1/10^5$
C 265 (from calf)	5th	1/400	1/400	n.o.	1/25	n.o.	1/50	,,	,,	$1/10^3$	$<1/10^3$	$<1/10^2$	$1/10^3$

C. = Crude elementary body suspension.
F.S_1. = Filtrate of first supernatant.
n.o. = Not obtained.
S_1, S_2, S_3 = First, second and third supernatants.
E.B. = Washed elementary body suspension.
N.t. = no test.
* Practically confluent but not completely so.

the absorption stage of the test the serum-" antigen " mixture represented a 1 in 5 dilution of the serum, so that the highest concentration in which the " absorbed " sera were examined represented a dilution of 1 in 10 serum. The mixtures of serum and antigen and a control mixture of serum and saline were allowed to stand for an hour at room temperature. They were then placed in a 55° C. incubator for a few hours, after which they were centrifuged at high speed. The supernatants were tested both for residual antibody and for residual antigen. The latter was detected by addition of antivaccinia serum, and when present in a dilution of the supernatant greater than 1 in 4 the tests were rejected. In testing for residual antibody, suspension and filtrate were employed in concentrations of four to eight times their titre. The tests were suffered and incubated in the same way as the flocculation tests described above. Cross-absorption tests were carried out with the filtrates and elementary body suspensions of V 138, V 140, V 143, V 145 and V 151 (Table III). In addition other tests were made in which filtrates obtained in earlier experiments were employed. Tables IV and V are representative of the results obtained.

It was found that elementary bodies absorbed not only their own agglutinin, but at the same time removed the precipitin for the Seitz EK filtrate. The filtrates, when applied in carefully adjusted doses, absorbed not only their corresponding precipitin, but the serum so absorbed failed to agglutinate the elementary body suspension.

These results demonstrate conclusively that the antibody in hyperimmune rabbit serum which precipitates the specific filterable flocculable substance of vaccinia extracts also agglutinates the elementary bodies of vaccinia. It seems permissible to conclude that the Seitz EK-filterable flocculable substance is a specific product of the elementary body.

TABLE IV.—*Absorption Test with Filtrate and Elementary Body Suspensions V 143.*

Treatment of serum §74.	Test antigen (or serum).	Dilutions of serum §74. 1 in 10.	1 in 20.	1 in 40.	1 in 80.	1 in 160.	1 in 320.	1 in 640.	1 in 1280.
Not absorbed	F.	n.t.	+++	+++	++	+	tr.	−	−
	E.B.	n.t.	+++	+++	+++	++	+	tr.	−
Absorbed with elementary body Suspension V 143	F.	−	−	−	−	−	−	−	n.t.
	E.B.	−	−	−	−	−	−	−	n.t.
	S.	−	−	−	−	−	−	−	n.t.
Absorbed with Filtrate V 143	F.	−	−	−	−	−	−	−	n.t.
	E.B.	−	−	−	−	−	−	−	n.t.
	S.	−	−	−	−	−	−	−	n.t.
Absorbed with Filtrate V 143	F.	++	+	tr.	−	−	−	−	n.t.
	E.B.	++	++	+	tr.	−	−	−	n.t.
	S.	−	−	−	−	−	−	−	n.t.

F. = Filtrate.
E.B. = Washed elementary body suspension.
S. = Serum (test for excess antigen).
+++ to + = Degrees of precipitation readily visible to naked eye.
tr. = Trace requiring confirmation with hand lens + 3.
− = Negative
n.t. = No test.

TABLE V.—*Summary of Absorption Tests with Filtrates V 108 and V 112.*

Treatment of serum §74.	Test antigen. F.V. 108.	F.V. 112.	E.B. 140.	E.B. 143.	E.B. 145.	Test for excess antigen.
Serum not absorbed, titre	1 in 160	1 in 160	1 in 320	1 in 320	1 in 320	n.t.
Absorbed with filtrate V 108, titre	<1 in 10	<1 in 10	<1 in 10	<1 in 10	<1 in 10	—
Absorbed with filtrate V 112, titre	<1 in 10	<1 in 10	<1 in 10	<1 in 10	<1 in 10	Trace 1 in 10 only.

CONCLUSIONS.

1. Two serologically active fractions may be obtained from extracts of lapine : (*a*) A Seitz (EK) filterable precipitable substance and (*b*) elementary bodies.

2. Both fractions are flocculated specifically by the same antibody of anti-vaccinia serum ; they do not react with normal rabbit serum.

3. The flocculation reaction, as performed with crude suspensions, involves both a precipitin and an agglutination reaction, referable to the same antigen in two different physical states.

4. A thrice-washed suspension of elementary bodies exhibits a virus content similar to that of the extract from which they were obtained.

5. These results warrant the conclusion that the Seitz-filterable flocculable substance is a specific product of the elementary bodies of vaccinia, and support the view of Paschen and others that these bodies are the virus of vaccinia.

REFERENCES.

BEDSON, S. P.—(1932) *Brit. J. Exp. Path.*, **13**, 65.

BURGESS, W. L., CRAIGIE, J., AND TULLOCH, W. J.—(1929) *Sp. Rep. Ser. Med. Res. Coun. Lond.*, No. 143.

CASAGRANDI, O.—Quoted by GILDEMEISTER, E., in 'Handbuch der Pockenbekämpfung und Impfung.' Berlin : Lentz and Gins, 1927, p. 741.

CRAIGIE, J., AND TULLOCH, W. J.—(1931) *Sp. Rep. Ser. Med. Res. Coun. Lond.*, No. 156.

EAGLES, G. H., AND LEDINGHAM, J. C. G.—(1932) *Lancet*, **1**, 832.

FREYER, M.—(1904) *Zbl. Bakt.*, Abt. I, Orig. **36**, 272.

GORDON, M. H.—(1925) *Sp. Rep. Ser. Med. Res. Coun. Lond.*, No. 98.

HAVENS, L. C., AND MAYFIELD, C. R.—(1931) *Amer. J. Pub. Hlth.*, **21**, 329.—(1932) *J. Inf. Dis.*, **50**, 242.

LEDINGHAM, J. C. G.—(1931) *Lancet*, **2**, 525.—(1932) *J. Path. Bact.*, **35**, 140.

NAUCK, E. G., AND PASCHEN, E.—(1932) *Zbl. Bakt.*, Abt. I, Orig., **124**, 91.

PASCHEN, E.—(1906) *Münch. med. Wschr.*, **53** (2), 2391.—(1913) *Deuts. med. Wschr.*, **39** (2), 2132.—(1924) In 'Lehrbuch der Infektionskrankheiten,' Berlin, Jochman-Hegler, 1924, p. 850.

SCHULTZ, E. W., BULLOCK, L. T., AND LAWRENCE, F.—(1928) *J. Immunol.*, **15**, 265.

TANAKA, K.—(1902) *Zbl. Bakt.*, Abt. I, Orig., **32**, 726.

THOMPSON, R., AND BUCHBINDER, L.—(1931) *J. Immunol.*, **21**, 375.

TULLOCH, W. J.—(1932) *Proc. Roy. Soc. Med.*, **25**, 453.

WOODRUFF, C. E., AND GOODPASTURE, E. W.—(1929) *Amer. J. Path.*, **5**, 1.—(1930) *Ibid.*, **6**, 713.

MUCUS SECRETION IN THE TRACHEA.

H. FLOREY,* H. M. CARLETON AND A. Q. WELLS.

From the Department of Pathology, Cambridge; and the Department of Physiology, Oxford.

Received for publication March 24th, 1932.

IN the trachea and bronchi there are two distinct sets of glands producing mucus. As shown in Fig. 1, goblet-cells are fairly numerous in the tracheal mucosa of the cat. Usually they give a typical red coloration with mucicarmine. Empty, collapsed goblet-cells, not giving this reaction, may often be observed. These cells are absent over the posterior tracheal fold.

There are found, in addition, acini of gland-cells lying in the submucosa. These cell-groups are serous, mucous or mixed in type. The submucosal glands, in contrast with the goblet-cells, have been shown to be innervated (Larsell, 1923).

These present experiments have had as their object the determination of the conditions in which mucus is discharged from these two groups of glands, and the observation of the histological changes involved in their activity.

EXPERIMENTAL.

Cats have been used almost exclusively for these investigations. The various methods employed will be described under the different experiments.

Effects of Stimulation of Nerves.

A straight tracheal cannula was inserted into a cat, anæsthetized by dial or chloralose, or decerebrated. One vagus nerve was dissected in the neck, tied and cut. During a control period of 3 hours it was observed that no obvious mucus collected in the cannula. The peripheral end of the vagus was then stimulated faradically, each stimulus lasting 1 1/5 seconds, with an intervening rest period of 1 second. At the end of a few minutes mucus secretion began to appear in the cannula, being pushed up from the trachea—presumably by the cilia. The secretion of mucus continued throughout the duration of the stimulus, and considerable quantities had to be evacuated from the cannula to prevent asphyxia. A method for measuring the amount of secretion obtained from a given length of trachea during a given time was devised. The technique of preparing the trachea and the methods of recording are fully described elsewhere (Florey and Wells, 1931). Briefly, a length of trachea is enclosed between two cannulæ, one of which is attached to a horizontal

* With a grant from the Medical Research Council.

graduated pipette by rubber tubing. The trachea is filled with oil, which extends to the beginning of the pipette. Any alteration of the volume of the enclosed trachea is recorded by an alteration of the reading of the oil in the pipette. This alteration can be due either to contraction of the tracheal muscle or to secretion of fluid into the tracheal lumen. Atropine will abolish the former when due to nervous influences or parasympathicomimetic drugs, so that the administration of this drug at the end of stimulation enables a separation to be made between secretion and contraction effects.

Preliminary experiments showed that when pure olive oil was used, the reading of the oil in the pipette usually remained remarkably constant during long periods. The administration of atropine or adrenalin did not alter this significantly. Disturbances such as swallowing had little effect.

The following are figures taken from an experiment in which the recurrent laryngeal nerves had been tied and cut low in the neck, and the peripheral ends subsequently stimulated for 1 minute alternately during the times given:

Time.	Reading of oil.	Increase. (1 div. = 0·2 c.c.)
12.44 p.m.	3·79	..
Stimulus.		
1.4	4·27	0·48
1.24	4·71	0·44
Left till 2.30. Level of oil brought back (as 5·00 was the end of scale).		
2.32	3·58	..
Stimulus.		
2.52	3·98	0·40
0·75 c.c. 1 per cent. atropine given. Stimulated again.		
3.12	4·02	0·04

By this means it was possible to measure the quantity of secretion from a given length of trachea over a given period of time, and to show the inhibition of secretion by atropine.

A further modification of this procedure was made by recording photographically the movements of the oil by means of the apparatus described elsewhere (Florey and Wells, 1931).

Fig. 6 is such a record of the amount of secretion obtained by stimulating for 1 minute the peripheral ends of the cut vagi alternately during 15 minutes. After the cessation of the stimuli atropine was given intravenously, which did not alter the oil level. A stimulation for a further period of 15 minutes produced no rise. Examination of the trachea post-mortem showed a healthy mucosa with a collection of clear mucoid fluid at the upper end. These experiments clearly show that mucus is secreted by the trachea when the vagus and inferior laryngeal nerves are stimulated, the effect being abolished by atropine.

Kokin (1896), working in Pavlov's laboratory, showed that drops of secretion could be seen on the dog's exposed trachea when the appropriate nerve was stimulated, and interpreted this observation as showing that tracheal mucus secretion was under the influence of the nervous system.

Reflex Stimulation of Glands.

After a control period of 1 hour the central end of one cut vagus was stimulated, in one experiment, for 1 minute, with an interval of 1/2 minute, for 55 minutes. A considerable amount of mucus secretion, first apparent after 15 minutes' stimulation, appeared from an incision made in the trachea. In another experiment the nerve was stimulated for 1 2/5 seconds with a rest period of 1 second for 1 1/2 hours. At the end of 10 minutes mucus began to appear, and continued to collect as long as the stimulus was continued. It was necessary to take particular precautions to keep the vagus warm and moist for the success of the experiment. This experiment thus shows the existence of centripetal fibres in the vagus, stimulation of which causes reflex mucus secretion.

Effect of Pilocarpine.

The trachea of a decerebrated or anæsthetized cat was opened longitudinally, and bleeding stopped by a small cautery. It was then opened out to obtain a view of the mucosa. This was carefully dried with filter-paper and an intravenous injection of pilocarpine given. Secretion could then be seen collecting in droplets on the surface of the trachea. This action of pilocarpine was abolished by atropine.

The secretion by pilocarpine was also measured photographically (Fig. 7). This experiment revealed the motor effects of pilocarpine on the tracheal muscle. In Fig. 7 the sudden upward rise of the oil level can be seen. This was sufficient to carry it off the drum. After the administration of six doses of 0·25 c.c. of 1 in 1000 pilocarpine, 0·20 c.c. of 1 per cent. atropine was given, which caused the level of the oil to recede owing to relaxation of the trachea muscle. The difference of levels between that at the beginning and that at the end of the experiment represents the amount of mucus secreted in the time. Also, after pilocarpine, droplets can be seen collecting on the surface of the trachea. This appearance is best interpreted by supposing that the fluid is coming from the ducts of the buried glands.

From results obtained on other similar glands, *e. g.* the salivary, it may be concluded that pilocarpine stimulated the same glandular elements as did the nerve supply.

Attempts in the cat to see the appearance of droplets on the surface of the trachea in response to nerve stimulation have given somewhat equivocal results, as the observations are not easy to make unless a pronounced change occurs—as is the case with pilocarpine ; Kokin (1896), however, observed this to be the case in the dog.

Are the goblet-cells similarly innervated, or do the two sets of mucus-producing cells react in different ways ? It has been shown elsewhere that the goblet-cells of the colon are not under nervous control (Florey, 1930). It was hoped that the photographic method of recording would be of assistance in settling this and other points. It might be reasonably assumed that stimuli from the mucous membrane of the trachea would activate at least the buried glands reflexly—the efferent side of the arc clearly exists. This reflex mechanism would be inhibited by atropine. If the goblet-cells were similar

to those of the colon they would not be influenced by the drug. Accordingly olive oil containing mustard oil was substituted for pure oil in the tracheal preparation. It was hoped to obtain a curve of a certain steepness due to secretion from the glands, which could be inhibited by atropine, and so produce a lessening of the rapidity of ascent of the curve. Unfortunately the contractability of the trachea interfered with the experiment.

It was found that the placing of an irritant in the trachea caused a steady rise of oil level, the rapidity being greater the stronger the mustard-oil solution. This rise was quite uninfluenced by cutting both vagi or by a moderate dose of atropine. A very large dose slowed the rise, but did not abolish it. The rise was not caused by secretion, because if a rapid, considerable rise were produced, and the animal killed and examined, there was always a totally insufficient amount of secretion present to account for the volume change. The production of œdema in the mucosa by the irritant would not account for the alteration of oil level, for some tracings were obtained in which the oil level made an almost vertical ascent, *i. e.*, a considerable reaction occurred within a few seconds.

It was thought that mustard oil might be a specific stimulant for the smooth muscle, so a solution of ammonia in serum was substituted, with, however, the same results.

Nevertheless, it was found possible to show the reverse side of the picture. Immediately after anæsthetization a cat was given 0·2 c.c. of 1 per cent. atropine and the tracheal pouch filled with oil (3 drops mustard oil in 50 c.c. olive oil). The oil was left in for 3 hours, when the animal was killed and the pouch evacuated. A core of thick elastic mucus had been produced with a certain amount of slightly pink fluid. From the presence of the latter it might be inferred that considerable damage to the mucosa had occurred. This, however, only appeared somewhat reddened, while no petechial hæmorrhages or other signs of severe tissue damage could be seen. This experiment was repeated several times with larger (up to 1 c.c.) doses of atropine, and with the same result.

When atropine was omitted a similar product was obtained, though perhaps there was more of it, but the quantities could not be accurately measured.

Another method was adopted which gave a similar result. By means of a ball-valve arrangement the inspired air bubbled through a 1·5 per cent. solution of ammonia (ammon. fort.) before passing to the lungs of a cat through a tube inserted between the vocal cords. The cords gripped this tube and made a good joint. When both vagi were intact a plentiful secretion of mucus appeared in the trachea. Even after cutting the vagi and large doses of atropine, secretion was still produced, though whether this was less in amount was difficult to determine.

Still further evidence will be given later that copious mucus secretion can occur after thorough atropinization. Thus it will be seen that the foregoing methods have failed to give unequivocal evidence that stimulation of the tracheal mucosa reflexly excites the buried glands to secrete. However, all forms of experiment agree in showing that a very considerable secretion may occur after the cutting of both vagi and after thorough atropinization.

It has not been possible to illustrate a reflex effect on the glands from a

local stimulation by the utilization of the photographic method of recording tracheal secretion. But, on the other hand, it can be definitely concluded from its use that irritation of the lower part of the trachea does not cause secretion in the upper part, *i. e.*, that no long reflexes exist between the lower and upper portions.

By the use of histological methods the following observations bearing on the above experiments have been made. The usual technique consisted in fixation in "Susa" (Heidenhain, 1915; 1917), and staining in Delafield's hæmatoxylin followed by muci-carmine.

(1) *Nerve stimulation of tracheal glands.*—The control to these observations was furnished by excision and immediate fixation of a segment of trachea before stimulation was begun. The deep glands in the stimulated trachea showed the classical signs of exhaustion, viz., reduction in the amount of histologically demonstrable intra-cellular mucin; the localization of the latter (when present) in the tips of the cells; its presence, usually in abundance, in the acini and ducts.

(2) *Pilocarpine stimulation of tracheal glands.*—After repeated doses of this drug, changes similar, but culminating in an almost complete disappearance of the mucus were noted. (See fig. 2.)

(3) *Goblet-cells.*—Experiments in which the nerves were directly stimulated have not given histological evidence permitting of definite conclusions. Successful preparations were, however, obtained after administration of pilocarpine, the goblet-cell content being increased, as a preliminary, by a method to be described later. Discharge of the goblet-cells did not seem to be in excess of the normal, although the tracheal glands were almost completely exhausted.

(4) *The effects of application of mustard oil.*—Diluted mustard oil was applied to a tracheal pouch in the manner described. Histological evidence was obtained showing that (*a*) the tracheal glands showed exhaustion similar to that obtained after nerve-stimulation or pilocarpine. (*b*) Clear-cut results were not obtained in regard to the goblet-cells owing to the tendency to desquamation of the superficial epithelium. (*c*) In the heavily atropinized subject exhaustion of the deep glands was very evident. In these preparations numerous discharging goblet-cells were also observed. (*d*) It was noted, in an animal in which both recurrent and superior laryngeal nerves had been sectioned that the goblet-cells were discharging, exhaustion of the deep glands being also present.

Dog Experiments.

It was thought that perhaps the larger trachea of the dog would make observations on the expulsion of secretion from the gland ducts easier to observe.

The trachea of a large dog, anæsthetized with chloralose, was opened in the midline by means of a cautery. It was then opened out so that the inner surface could be observed. After trying various procedures calculated to produce a reflex secretion, the conclusion was reached that the method of visual observation was too hazardous for reliable conclusions to be drawn. The only unmistakable case in which secretion could be seen rising from the ducts was after the application of a faradic current to the mucosa, but as it was impossible

to separate satisfactorily a reflex effect from one directly acting on the nerves to the glands, it was decided to abandon this method of approach.

It would be reasonable to assume that the function of the glands is to react to some slight stimulus, *e.g.*, the presence of a foreign body in the trachea, so as to aid its expulsion, or that they are primarily concerned in keeping the trachea moist—a function similar to that of the glands of the mouth.

The following experiments were done on the assumption that such would be the case.

Cats prepared with tracheal pouches and two cannulæ as already explained were used. By means of either a water-suction pump or an electric blower, a stream of air was passed over the mucous surface of the trachea. This stream was first thoroughly dried. The most satisfactory arrangement was to pass the air over $CaCl_2$, firstly, and finish the drying over P_2O_5. After passing this perfectly dry air over the trachea, the moisture it took up therefrom was collected either in weighed $CaCl_2$ tubes, or, in what proved to be better, in tubes containing P_2O_5. It was thus possible to find the amount of water given off during stated time-intervals.

If the glands therefore were stimulated to produce fluid this would be evaporated, and the amount of water collected would be a measure of the amount of secretion poured out. If atropine were given (which paralyses the influence of the nerves on the deep glands), then the amount of water collected in unit time should show a decided fall, providing the protection of the tracheal surface against desiccation depended largely on the buried glands.

The surprising result was consistently obtained that the amount of water collected showed a very slight or no diminution after the administration of large doses of atropine.

For instance, dry air blown through trachea for 3 minutes and then P_2O_5 tubes attached to outlet :

Results : 1st 5 mins. weight water collected, 0·104 gm.
2nd 5 ,, ,, ,, ,, 0·130 ,,
3rd 5 ,, ,, ,, ,, 0·100 ,,

0·5 c.c. of 1 per cent. solution atropine sulphate given intravenously immediately.

Interval of 1 minute :

Results : 4th 5 mins. weight water collected, 0·109 gm.
5th 5 ,, ,, ,, ,, 0·113 ,,
6th 5 ,, ,, ,, ,, 0·202 ,,

The trachea was opened immediately and found to be dry. Air had been passed at the rate of 1 litre in 46 secs.

The following longer experiment indicated the steady rate at which the trachea can give off water.

1 hour { 1st 15 mins. ; water caught, 0·243 gm.
2nd ,, ,, ,, 0·251 ,,
3rd ,, ,, ,, 0·261 ,,
4th ,, ,, ,, 0·266 ,,

2 hours	5th	15 mins. ;	water	caught,	0·283	gm.
	6th	,,	,,	,,	0·265	,,
	7th	,,	,,	,,	0·252	,,
	8th	,,	,,	,,	0·260	,,
3 hours	9th	,,	,,	,,	0·266	,,
	10th	,,	,,	,,	0·248	,,
	11th	,,	,,	,,	0·262	,,
	12th	,,	,,	,,	0·268	,,

When examined the trachea was covered with a small amount of mucus secretion, except for one small patch which appeared dry. The rate at which the air passed through was not measured.

The following experiment shows a very small drop following the administration of atropine:

	1st	15 mins. ;	water	caught,	0·235	gm.
	2nd	,,	,,	,,	0·255	,,
	3rd	,,	,,	,,	0·232	,,
	4th	,,	,,	,,	0·233	,,
0·3 c.c. atropine 1 per cent. given :						
	5th	15 mins. ;	water	caught,	0·232	gm.
	6th	,,	,,	,,	0·193	,,
	7th	,,	,,	,,	0·209	,,
	8th	,,	,,	,,	0·210	,,
	9th	,,	,,	,,	0·200	,,
	10th	,,	,,	,,	0·218	,,
	11th	,,	,,	,,	0·205	,,
	12th	,,	,,	,,	0·204	,,

On examination the trachea was covered with a moist coat, except for one small patch which appeared dry.

Further observations were made by similar methods. If the dried air were run over the trachea at the rate of 1 litre in 41 seconds, at the end of 1/2 hour, if not sooner, the trachea of an atropinized cat appeared dry. If, however, the cat were left for 1 hour after the cessation of the passage of the air and then examined, it was found that the trachea was covered by a thick coating of moist mucus. Clearly this abundant mucus secretion had gone on in spite of the atropine.

Histological examination.—The prolonged passage of dry air was found to be definitely harmful to the epithelium, desquamation being elicited. Such goblet-cells as were still *in situ* were found to be actively discharging. The tracheal glands were swollen, this being due to swelling of the constituent cells as well as to dilatation of the acini. The mucin content of the cells was increased. The submucosa was œdematous and its blood-vessels dilated.

It would thus appear that the air blast causes an acute inflammation, the bulk of the water collected, however, being the product of vascular transudation rather than secretion from the glands.

A modification of the above experiment was also used. The trachea of a cat was incised in the midline by means of a cautery and opened for inspection.

EXPLANATION OF FIGURES.

All figures drawn with camera lucida at a magnification of 455 diameters. Present magnification, × 365, owing to reduction. All figures on these plates are from sections of trachea of cat (except No. 3). Fixation in "Susa"; Delafield's hæmatoxylin and muci-carmine. B., Blood-vessels in submucosa; C., columnar epithelial cell; D., duct of tracheal gland; G., goblet-cell; M., mucus cell; P., polymorph leucocyte; S., serous cell; X, see explanation of figure in question.

FIG. 1.—Normal trachea showing goblet-cells containing mucus, and two in process of discharging it. A few of the serous cells contain intra-cellular mucus (x), which is present in all the cells of the mucous alveolus depicted.

FIG. 2.—After injection of pilocarpine. The mucus of the goblet-cells stains faintly, and is not appreciably reduced in amount; the gland cells are completely exhausted (except at x); some mucus in their lumina.

FIG. 3.—From a case of chronic human bronchitis. Note the almost complete substitution of large goblet-cells in place of the columnar elements; the former all contain histochemically detectable mucus and most of them are actively discharging it.

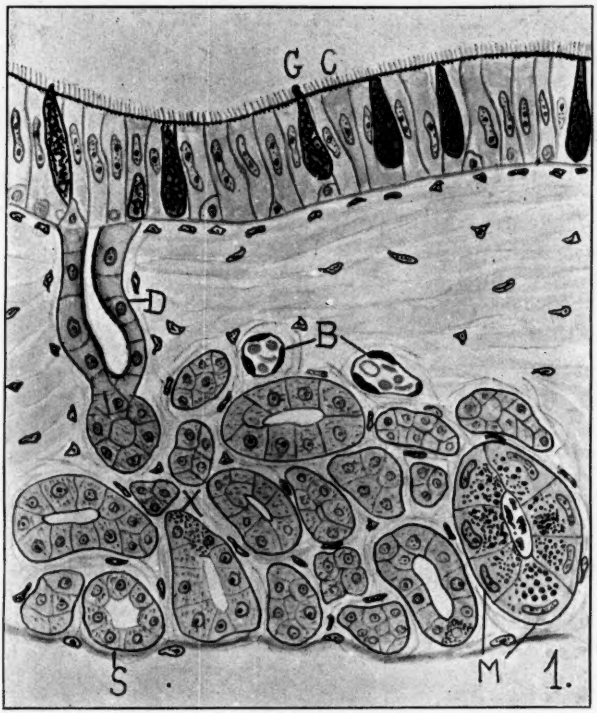
FIG. 4.—Intra-tracheal injections of 3 c.c. daily with 1/8 per cent. formol for 9 days. This has provoked a very marked increase in the number of goblet-cells; they also tend to be unduly large, and contain mucus, which is being discharged. The deep glands, here "serous" morphologically, contain intra-cellular mucus (x), but show no signs either of discharge or exhaustion.

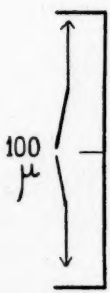
FIG. 5.—Daily intra-tracheal injections of 1/4 per cent. formol (of 5 c.c. each) for 7 days. Owing, presumably, to the stronger concentration used, the columnar epithelium has been replaced by one of the stratified type. It is rather similar to the transitional epithelium of the urinary bladder. Mitoses (x) are abundant. The deep glands contain no mucus and are clearly exhausted; there is a little in the alveoli and ducts.

FIG. 6.—Photographic record of secretion produced by alternate stimulation of both vagi. The rise in the curve is due to the accumulation of mucus, as there is no fall after the administration of atropine, which relaxes muscular contraction. There was no further secretion of mucus on stimulation after the dose of atropine. The horizontal lines represent a volume of 0·01 c.c.

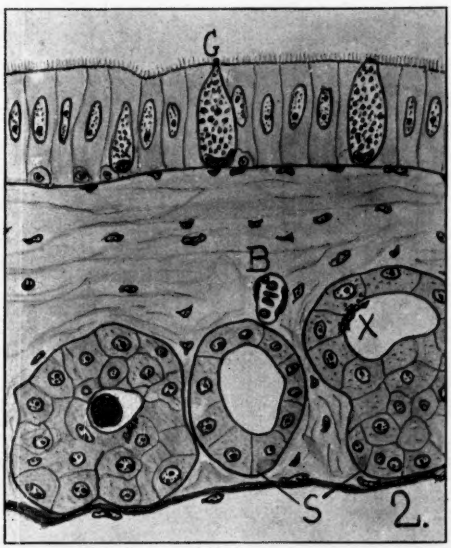
FIG. 7.—Effect of administering successive doses of 1 : 1000 pilocarpine. There is a rapid muscular contraction which carried the record off the sensitive paper. On the administration of atropine there was a rapid relaxation of the contracted muscle. The difference between the level at the beginning and after atropine administration represents the amount of mucus secreted in the time. For purposes of reproduction the curve has been outlined in Indian ink and the photograph bleached.

FIG. 8.—*a*, Low-power photograph of a "patch" of small intestine removed at biopsy after being *in situ* 2 years. Note absence of villi, but very long crypts of Lieberkühn. The black substance beginning in the tips of the cells towards the bottom of the crypts and gradually increasing towards the surface is mucus stained red by muci-carmine. *b*, High-power photograph of "patch" stained by Masson's aniline blue-ponceau-acid fuchsin. This is a portion of the patch showing the enormous increase of the number of goblet-cells present.

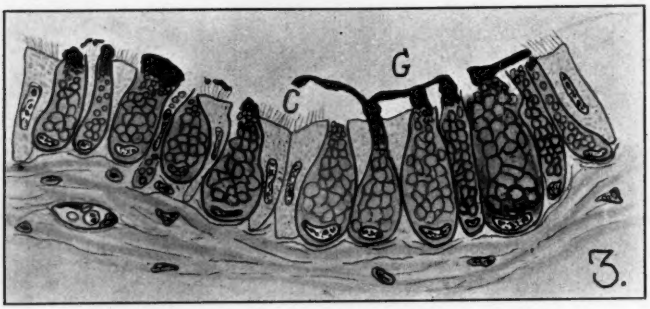


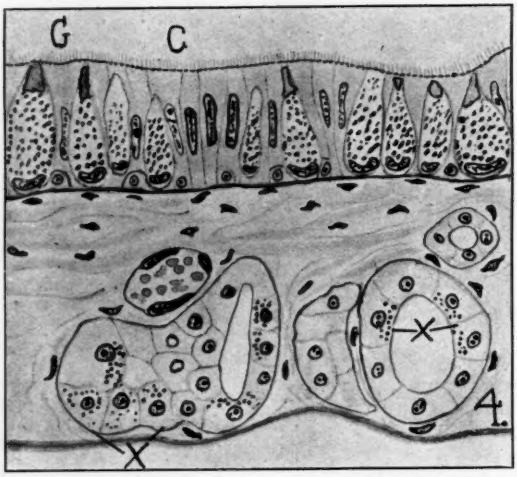


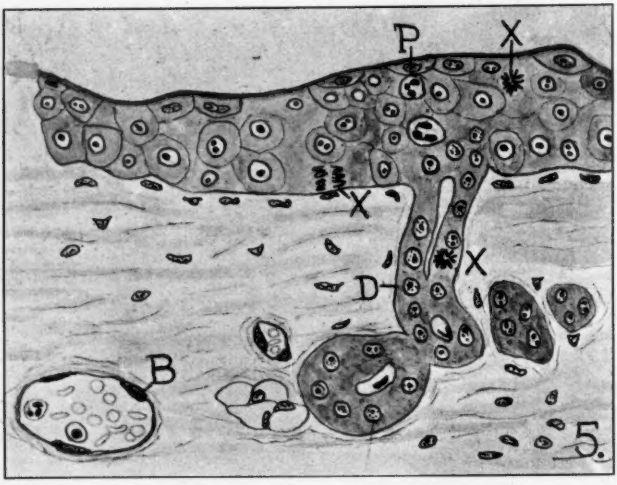
H.M.C.
del..



Florey, Carleton and Wells.







Florey, Carleton and Wells.

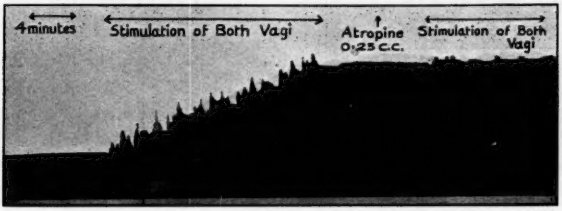


FIG. 6.

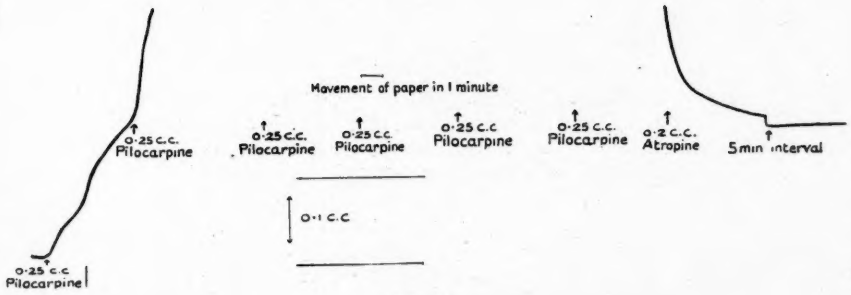


FIG. 7.

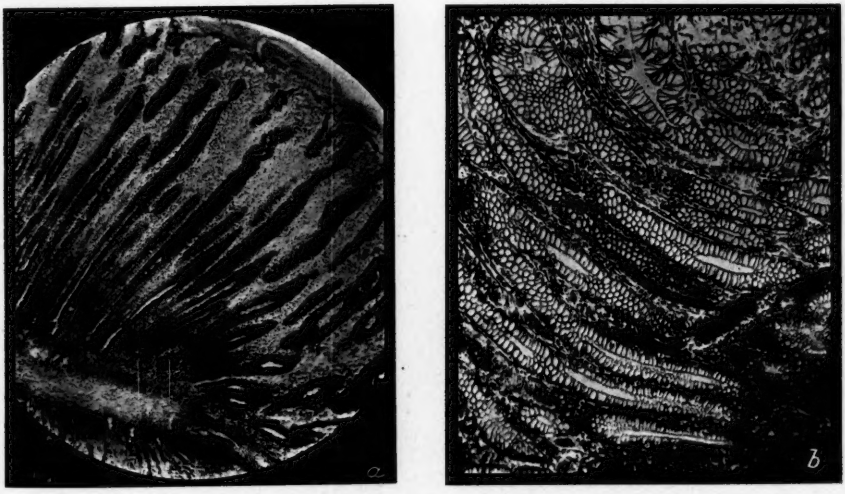


FIG. 8.

A concentrated beam from a "pointolite" was so adjusted that a bright reflection was obtained from a portion of the moist mucosa. By directing a blast of dry air against this spot the moisture was rapidly removed and the mucosa then presented a matt surface. The time was then taken by a stop-watch for the glistening appearance to return.

Atropine was then given intravenously and even applied locally and the time again noted.

The following figures were obtained:

Intervals for reappearance of glistening surface, 8 secs., 9 secs., 9 secs.

0·5 c.c. 1 per cent. atropine injected intravenously:

After 5 minutes' interval: 8 secs., 9 secs., 8 secs.

Clearly no difference.

A further modification of this type of experiment was as follows: A slow stream of dry air was passed through a tracheal pouch, which at the end of an hour was excised and examined. The mucosa was moist, but there was no free fluid or mucus. In other cats the air was bubbled through ether before being dried. At the end of an hour there was an abundant collection of free fluid containing mucus inside the pouch. The experiment was then repeated, giving as a preliminary 0·4 c.c. of 1 per cent. atropine intravenously, but there was still an abundant collection of rather thin mucoid fluid. The administration of the atropine had made no appreciable difference to the amount of fluid present. The fluid produced by the ether irritation was much thinner and more watery than that produced by other forms of irritants tried. It was quite clear, and showed no macroscopical evidence of being produced by gross damage to the mucosa. Histological examination of specimens from these experiments gave the following results:

Normal trachea.—There were goblet-cells in moderate numbers—mostly faintly positive with muci-carmine. The glands showed a fair proportion of cells containing mucin. There was a little positive secretion, some of it in the ducts, many of which were dilated.

Trachea treated with ether.—The general appearance is much the same as the above, but the proportion of positively stained gland-cells is apparently smaller than in the normal. There is so great a regional variation in the distribution of the goblet-cells that it is difficult to make any comparison between this and the normal specimen. In some places there is a thick layer of material, negative for mucin, lying against the epithelium. It contains some oval bodies which stain like mucus, and which might be the contents of goblet-cells still retaining the shape of the cells.

In another experiment, 0·4 c.c. of 1 per cent. atropine was given intravenously and the animal killed at the end of 40 minutes.

Normal trachea.—General condition similar to that of the preceding experiment.

Trachea treated with ether.—Condition as in control trachea.

In another similar experiment the trachea treated with ether showed a fair number of feebly positive goblet-cells, the gland-cells being mostly negative, their lumen dilated.

Thus, histologically, although a very copious secretion is called forth by ether, there is no very good evidence to show that the goblet-cells are emptied.

The thin secretion noted macroscopically from its staining reactions does not appear to contain much mucus. Inasmuch as this fluid appears whether the deep glands are paralysed by atropine or not, it would seem that it must come largely from the tracheal surface, though the goblet-cells do not appear to be particularly involved. On the other hand, some histological evidence was obtained that the deep glands secrete when stimulated by the ether, but that this is inhibited by atropine.

Support for this view can also be obtained from observations on an opened trachea dried by an air-blast after thorough atropinization. After drying, the surface rapidly becomes moist again, but this fluid is "thin," and does not appear to be very mucoid—a fact better observed microscopically in the way to be described.

Microscopical Observations in vivo.

The trachea of a cat was opened in the midline by a hot knife. This was found to cause a minimum of damage, and saved considerable trouble in stopping bleeding from cut vessels after a scissors cut. The trachea was held apart sufficiently for observation by small hooks. The source of light came from a 100 candle-power "pointolite," while a binocular dissecting microscope was used for observation. When observed with a magnification of approximately 25 diameters the blood supply of the mucosa was readily seen. Where the light was sharply reflected from the tracheal surface the activity of the cilia could be clearly observed. Where the light was more diffusely reflected, what seemed under the microscope to be a "river" of secretion could be seen rapidly passing along the trachea towards the larynx. This constantly moving layer made it impossible to distinguish where the secretion was coming from.

Various procedures were adopted to see if this could be determined. A blast of dry air was directed on to the trachea till it appeared quite dry and matt. On removal of the blast the mucosa rapidly swelled again and the surface became moist. This procedure was repeated many times, but it was never possible to separate clearly the contribution of the tracheal surface and the deep glands to this moisture. The moisture appeared at first to come evenly from all the surface. After the lapse of some time large oval collections on the general moist surface appeared. These perhaps represented secretion issuing from the deep glands. A point previously mentioned as to the rather watery character of this secretion could be well appreciated by touching the secretion with a needle—it did not adhere. Mucoid secretion, such as was normally present before interference, or after pilocarpine, could be pulled into strings. After atropine no clear-cut differences were detectable, the rather watery secretion appearing as before. It might be thought that these short blasts of air inflicted severe damage on the mucosa, but such did not appear to be the case. When dried the ciliary action could be seen to cease, but with the return of the moisture it once more appeared. It was only after repeated dryings that the cilia permanently ceased to function.

When a small drop of suspension of graphite ink (hydrokollag 300) in normal saline was applied to the trachea, the following picture of the clearing action of the cilia was very striking: The graphite particles adhere to the strands of mucus as they are being propelled along the trachea. Some of the particles

sink down and tend to remain, or at least are rather slowly moved forward until they are caught on the strands of mucus. These latter tend to be rolled up as they pass along, the process being assisted by the elasticity of the mucus. The strands appear to stick in some portion of their length from time to time, while the more forward portions are propelled onwards by the cilia. The force of the latter is sufficient to rupture the mucus strands, which then tend to roll up owing to their elastic recoil.

As Leonard Hill (1928) has shown on excised tracheæ, this power to remove small foreign bodies is very marked. From our observations the ability would appear to depend to a certain extent on the "stickiness" of the mucus. Another point which emerged was that when a considerable quantity of mucus secretion was present, the mucus stream moved more quickly than when the normal "resting" secretion alone was present. For instance, after a pilocarpine injection, the rate of the passage of the mucus stream was considerably accelerated.

Another attempt was made to utilize the above observations to demonstrate the reflex activity of the deep glands. If a dry substance is placed on the mucosa this has first to be thoroughly moistened by secretion before it is removed by the cilia. For these observations fine starch powder was found to be most suitable, though animal charcoal gave the same results.

The following figures were obtained in such an experiment. A piece of trachea only 3 cm. long was used, the portion below being isolated by destroying an intervening strip of mucosa. Times from the application of the starch particles to the first move were taken by stop-watch, the observation being made with the binocular microscope. A, B, etc., represent separate applications of starch:

	A.	B.	C.	D.	E.	
Minutes	2′ 30″	3′	2′ 30″	2′	2′	First move noted.
	6′	4′ 45″	3′	5′	3′ 45″	All particles moving.

1 c.c. of atropine was then given and 10 minutes allowed to elapse:

	F.	G.	
Minutes	3′	2′ 30″.	Started to move.
	4′	4′ .	All particles moving.

In this experiment a particle was observed to travel 3·5 cm. in 1 minute. This experiment was repeated with the same results, *i. e.* the trachea was found to be able to move dry particles equally well whether the deep glands were atropinized or not.

It was observed in some experiments that after atropine there appeared to be a decrease in the amount of secretion passing across a field though there was still an adequate flow. (Care was taken to see that the flow only came from the portion of trachea observed by destroying the mucosa below it, either with the cautery or by scraping with the scalpel.)

An attempt was made to make this observation quantitative by catching the stream of mucus at the end of its journey. Small pieces of blotting-paper about 1½ mm. wide were pressed on to the space extending between the posterior fold and the cut edge of an opened trachea, to collect the secretion

forced up from about 3 cm. of trachea. These pieces were replaced from time to time, the thick mucus collected against the edge of the blotting-paper being assisted on to it by a fine glass rod.

These pieces were weighed before and after the experiment in covered watch-glasses to avoid evaporation.

Protocol of such an Experiment.

1st watch-glass in 15 mins, ·0075 gm.
Animal then given 1 c.c. 1% atropine intravenously.
20-minute interval:
2nd watch-glass in 15 mins., ·0055 gm.
3rd ,, ,, ·0014 ,,

There is a distinct drop in the amount of secretion, but the quantities collected are very small. This small amount of secretion gives the impression microscopically of a moving layer of mucus of some considerable thickness.

An attempt to measure the secretion was made by applying a small piece of blotting-paper to the trachea, and noting the time taken for it to appear uniformly moist. It was thought that if the deep glands were contributing any considerable amount of secretion under these conditions, then, on giving atropine, it should take longer for the paper to get moist. Care was taken to shield the piece of paper from the current of secretion constantly travelling up the trachea.

Results of an Experiment.

Times taken for paper to become completely moist (each a separate application):
1′; 2′; 2′ 50″; 2′ 30″; 2′ 50″; 2′ 30″; 2′ 30″; 2′ 30″;
1 c.c. of 1% atropine given:
3′; 2′; 2′ 30″.

Here again, therefore, the contribution of the deep glands did not seem to be very great.

That, however, the deep glands are constantly contributing to the tracheal secretion was seen in some experiments with the use of graphite ink.

Thin streams of mucus issuing from a single point could be seen to stand out against the dark background of the ink, much in the same way as spirochætes do in an Indian ink preparation. These streams were not observed after atropine.

After a dose of pilocarpine large eruptions of a similar nature were observed, especially if the cilia were first killed by some bromine vapour.

These observations could not be satisfactorily extended owing to the difficulty in controlling the conditions so as to keep them constant over a period of time.

It is thus abundantly clear from these experiments that the surface of the trachea can become moist very easily when subjected to any stimulus employed, without the participation of the deep glands. Nevertheless, it must be admitted that any stimulus which it has been practicable to use is probably more vigorous

than those to which the trachea is normally exposed, approximating more to pathological conditions such as a bacterial tracheitis.

DISCUSSION.

It appears certain from the physiological and histological results taken in conjunction, that stimulation of the appropriate nerves activates the tracheal glands. Exhaustion of these can, in fact, be brought about by sufficiently prolonged stimulation. This contention is strongly supported by the results of pilocarpine injections, which produce considerable secretion, followed by microscopically demonstrable exhaustion of the glands. The secretion caused by both these methods is inhibited by atropine.

No satisfactory evidence has been forthcoming that the goblet-cells can be discharged by nerve-stimulation. Even the administration of pilocarpine in doses sufficient to exhaust the tracheal glands leaves the goblet-cells not appreciably affected. Attempts by several methods to demonstrate the reflex activation of the tracheal glands have not furnished any conclusive evidence, but direct stimulation of the central end of the vagus will cause secretion.

The application of dilute mustard oil has been shown to cause microscopically demonstrable exhaustion in animals (*a*) with all nerves intact, (*b*) with both the recurrent and superior laryngeal nerves sectioned, and (*c*) after the administration of massive doses of atropine. We therefore conclude from these experiments that activity of the tracheal glands is due to direct action of the irritant upon the gland-cells. Some evidence was obtained that the goblet-cells were partly discharged by the mustard oil, though complete exhaustion was never noted.

The passage of dry air caused inflammation and the production of considerable amounts of fluid, which, however, did not seem to be formed by glandular activity. This fluid appears to come directly from the vessels.

Experiments in which dry ether vapour was passed slowly through the trachea were found to produce less irritation than dry air passed more rapidly. Here some microscopical evidence was obtained of the inhibition of the tracheal glands by atropine.

A striking feature of our observations is the ease with which the surface of the trachea can produce fluid without any significant intervention on the part of the glands. It is to this that we attribute our inability to demonstrate satisfactorily reflex activation of the glands from stimulation of the tracheal surface. We consider, for this reason, that experiments such as those performed by Rossbach (1882) and Calvert (1896) are of limited value, in that the open trachea was first dried with blotting-paper and the time to get wet again noted. Rossbach and Aschenbrandt (1881) noted that, after section of the nerves and clamping of the trachea in two places, the degree of secretion remained unaltered. They concluded that little secretory activity (if any) was elicited through the nervous channel. This inference seems to be quite unjustified owing to the experimental difficulties which we have indicated.

A point of histological interest is that the hard and sharp division of gland-cells into mucous and serous elements would appear to be open to doubt. Thus, cells and acini, which on a purely morphological basis would be called

serous, proved, after treatment with specific stains for mucus, to contain this substance. Actual discharge was also noted.

THE EFFECT OF CHRONIC IRRITATION ON THE NUMBER OF GOBLET-CELLS.

Human bronchitis.—Cases of this were investigated. Sections were made from 14 of these and stained by the modification of muci-carmine described below. This was essential, as application of the ordinary method gave very poor results. Post-mortem change may possibly account for this.

Sections were mordanted for 10 minutes in a 0·5 per cent. solution of aluminium chloride in 50 per cent. alcohol. They were then stained for 1 hour in a 1 in 3 to 1 in 4 solution of muci-carmine in tap-water.

Of the 14 bronchi examined the epithelium was lacking in 5 ; in some cases this was due to acute inflammation having made it slough ; in one or two, post-mortem desquamation appears to be the cause.

In one specimen there was no definite increase in the goblet-cells, though the deep glands were undoubtedly highly active.

In the remaining 8 the goblet-cells were greatly increased in numbers. In 2 specimens the major part of the mucosa was constituted solely of these cells (see Fig. 3). Many were in process of extruding a plug of mucus. The deep glands showed dilated alveoli, the cells and lumina of which contained strongly positive mucus. The majority of these cells were mucous in type, but elements which, on a purely morphological basis, would be unhesitatingly termed " serous," contained a secretion, usually granular, which readily stained with the specific mucin stain.

One curious feature remains to be noted : A number of the columnar cells of the ducts contained mucus and appeared to be contributing it to the general production.

To sum up : In all cases (except one) in which the mucosa was sufficiently intact for estimation to be made, a widespread substitution of goblet-cells for columnar cells was obvious. So also was the great activity of the deep glands. The scarcity of " serous " cells in many of these sections, when taken in conjunction with the frequent presence of mucus in these elements, suggests that they are capable of elaborating mucin.

Thus in bronchitis, not only is there evidence of greatly increased production of mucus by the buried glands, but the ciliated surface epithelium itself may become almost totally replaced by cells of the goblet type.*

The effects of intratracheal injections of formalin were investigated. The concentrations used were 1/4 and 1/8 per cent., the duration varying from five to fourteen daily injections of 3 c.c. each. The technique was as follows : The trachea was injected under deep ether anæsthesia. A good view of the glottis was obtainable by opening the jaw with the animal supine and the head fully extended. A long glass tube, to which a syringe was attached, was then passed through the glottis for a distance of 3 cm. The animal was held head up while the formalin was forcibly injected.

* We are indebted to Dr. W. D. Newcomb, of St. Mary's Hospital, and also to Dr. Baker, of the Middlesex Hospital, for these specimens.

Two main effects were noted: firstly, the epithelial cells (Fig. 5) lost their columnar shape and cilia, and became transformed into polygonal elements rather similar to the transitional epithelial cells of the urinary bladder. (This was particularly noticeable after injections of 1/4 per cent. formalin.)

Secondly, there was an enormous increase in the number of goblet-cells as compared with normal cats (Fig. 4). In some sections, indeed, they greatly outnumbered the columnar cells. From the fact that many of these cells are deeply situated and abut on the basal layer, we conclude that they are probably derived from the undifferentiated cells of this region.

The glands generally showed much mucin, both in the cells and in the lumina, though in some cases there was evidence that their enhanced activity had resulted in exhaustion.

Observations on the bronchi of guinea-pigs subjected to dust inhalation (Carleton, 1924; 1927) also revealed an increase in the number of goblet-cells. In cases where the bronchitis was marked, not only is the epithelium largely composed of these elements, but large numbers of them lie in the plugs of *débris* in the lumina of the bronchi.

Chronic irritation of the small intestine.—A "patch" of ileum was inserted on to the surface of the abdominal wall in a dog with the same technique as previously adopted for the colon (Drury, Florey and Florey, 1929). A lateral anastomosis was used. Biopsy of the mucosa of the patch was made two years after the insertion of this patch. Microscopically it was noted that the villi had disappeared, the mucosa consisting solely of deep crypts. Concomitantly with this the number of goblet-cells was enormously increased (Fig. 8). These changes are attributable to chronic irritation induced by exposure of the mucosa, as evidenced by a slight leucocytic infiltration.

It would thus appear, from the four types of observations quoted, that chronic irritation of a mucous membrane normally provided with goblet-cells results in a striking increase of these elements.

SUMMARY.

1. Evidence has been produced to show that the glands of the trachea are activated by stimulation of the recurrent laryngeal nerves.

2. Pilocarpine causes similar activity.

3. The secretion provoked by both 1 and 2 is inhibited by the administration of atropine.

4. Stimulation of the central end of the vagus causes these glands to secrete reflexly.

5. Owing to technical difficulties we have been unable to obtain satisfactory evidence of reflex secretion caused by direct stimulation of the mucosa. We regard this, however, as a probability.

6. The immediate effects of various types of irritant are described.

7. Chronic irritation of mucosæ such as those of the trachea, bronchi and small intestine, leads to a great increase in the number of goblet-cells. In the case of the trachea more intense irritation causes the columnar and goblet-cells to be replaced by a stratified epithelium rather similar to that of the urinary bladder.

8. Gland-cells which on a morphological basis would be termed "serous" appear often to be capable of elaborating mucus, as evidenced by their histochemical reactions.

We are indebted to the Government Grants Committee of the Royal Society for a grant towards our expenses.

REFERENCES.

CALVERT J.—(1896) *J. Physiol.*, **20**, 158.
CARLETON, H. M.—(1924) *J. Hyg.*, **22**, 438; (1927) *ibid.*, **26**, 227.
DRURY, A. N., FLOREY, H., AND FLOREY, M. E.—(1929) *J. Physiol.*, **68**, 173.
FLOREY, H.—(1930) *Brit. J. Exp. Path.*, **11**, 348.
Idem AND WELLS, A. Q.—(1931) *J. Exp. Pharmac. Ther.*, **42**, 133.
HEIDENHAIN, M.—(1915) *Z. wiss. Mikr.*, **32**, 232; (1917) *ibid.*, **33**, 232.
HILL, L.—(1928) *Lancet*, **2**, 802.
KOKIN, P.—(1896) *Arch. ges. Physiol.*, **63**, 622.
LARSELL, O.—(1923) *J. Comp. Neurol.*, **35**, 97.
ROSSBACH, M. J.—(1882) *Berl. klin. Wschr.*, 281, 302.
Idem AND ASCHENBRANDT, T.—(1881) *Mschr. Ohrenheilk.*, No. **3**.

STUDIES IN *B. PESTIS* ANTIGENS: I. THE ANTIGENS AND IMMUNITY REACTIONS OF *B. PESTIS*.

HARRY SCHÜTZE.

From the Bacteriological Department, Lister Institute, London.

Received for publication May 7th, 1932.

MANY years ago Rowland (1914) made the observation that *B. pestis*, when grown at 37° C., developed a gelatinous envelope which he demonstrated by means of film preparations in indian ink.

A tiny loopful of agar culture or of the centrifuged deposit of a broth culture, grown at 37° C., is mixed with an equally small loopful of suitable indian ink so that the resulting film shall be a thin one, and is examined under a coverslip by the oil-immersion lens. Each plague germ is seen to be surrounded by a clear zone of considerable extent—the gelatinous envelope. Similar preparations made from cultures grown at 20° C. show no envelopes; the oscillating particles of indian ink directly bombard the naked bodies of the bacteria. At 26° C. there will be a meagre development in the case of virulent passage strains, while with old non-virulent laboratory strains it is not until a still higher temperature is reached that envelopes begin to appear—a fact which is of interest when correlated with the lack of prophylactic value possessed by non-virulent cultures. At 37° C., however, the phenomenon is, in the author's experience, a constant one; as, at this temperature, envelopes develop upon

all plague strains, no matter what their degree of virulence or their provenance, and are never to be found in the so closely related *B. pseudotuberculosis rodentium*, this feature should serve for the quick and certain diagnosis of the plague bacillus (*vide* Fig. 1).

Rowland distinguished this envelope from the capsule by the fact that there was no definite limiting membrane; he was not able to stain the envelope; he considered it to have rather the nature of an exudate, so that a chain of bacilli does not consist of a series of separately encapsuled bacteria, but of a row of organisms embedded in a continuous mass of the substance.

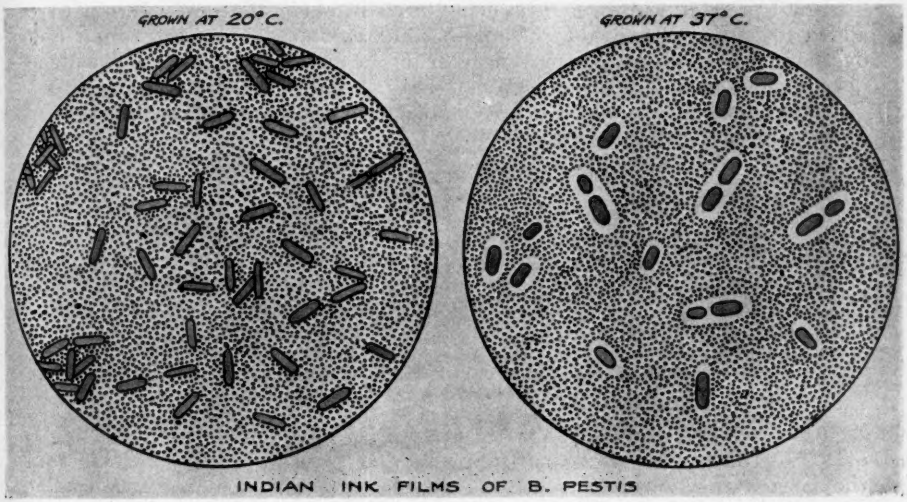


FIG. 1.

This observation of Rowland's has not received much attention, and its significance has never been properly appreciated.

It now appears that the gelatinous envelope contains a very definite antigen which is of great importance in immunity processes.

PRECIPITIN REACTIONS OF B. PESTIS.

For the study of the precipitin reactions of *B. pestis*, the use of antigens and sera corresponding to the gelatinous envelope on the one hand and to the body of the bacillus on the other was aimed at. It was found that heating 37°-grown plague bacilli, suspended in saline, at 60° entirely dissolved the envelopes, and that the supernatant of a heated suspension of this sort contained a large proportion of envelope substance and a small one of somatic substance. On the other hand, by suspending a 26°-grown agar culture in saline, killing at 60° and centrifuging, a supernatant was obtained which contained a large

proportion of somatic substance and a small one of envelope substance. It was then discovered that, while the envelope substance was heat-labile, that of the soma was stable. A short steaming of ¼ hour will haptenize the envelope antigen, so that while it will still precipitate with its corresponding antibody, it is quite inert as an immunogen in the animal body. Steaming for 1 hour destroys it for *in vitro* reactions as well, while the somatic antigen withstands this and is still able to precipitate with its appropriate antibody.

With the aid of this knowledge it was possible to prepare sera containing on the one hand mainly envelope antibodies, or, on the other, only somatic antibodies.

In all precipitin tests, five capillary drops of a 1/5 dilution of serum were added to two capillary drops of antigen, mixed, stood at 37° for 1 hour and overnight in the cold room. The criterion of a positive test was the development of a deposit and not an opalescence.

The following table (I) will give qualitative and quantitative information of the antigens employed:

TABLE I.

Serum.	Antigen.	Antigen titre.
Rabbit inoculated with envelope antigen	Envelope .	1/64
	Somatic .	<1/1
Rabbit inoculated with somatic antigen	Envelope .	1/4
	Somatic .	1/64

Envelope antigen consists of the supernatant of a suspension of *B. pestis* grown at 37° (1 slope of a 2-day culture per c.c. saline) centrifuged after heating to 60° for 1½ hours).

Somatic antigen consists of the supernatant of a suspension of *B. pestis* grown at 26° (2 slopes of a 3-day culture per c.c. saline) centrifuged after heating to 100° for 1 hour.

Absorption of sera (two slopes of culture per c.c. of 1/5 serum for 2 hours at 37° C.) differentiates the two antigens and their respective antibodies in the same way. Culture grown at 26° has so little envelope substance that it will only deplete a somatic serum and not an envelope serum; grown at 37° it will remove precipitin from both types of sera.

It has already been mentioned that the two antigens differ in their resistance to heat. In Table II an indication of the extent of these differences is given:

TABLE II.

	Antigen dilutions.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.
Anti-envelope serum	Envelope antigen .	++	++	++	++	++	+	+
	Ditto heated ½ hour 100° C. .	++	++	++	++	+	+	−
	Ditto heated 1 hour 100° C. .	−	−	−	−	−	−	−
Anti-somatic serum	Somatic antigen .	++	++	++	++	++	++	+
	Ditto heated 1 hour 100° C. .	++	++	++	++	++	+	tr.
	Ditto heated 3 hours 100°C. .	++	++	++	++	+	+	tr.

Envelope antigen remains active *in vitro* after ½ hour at 100°, although animal inoculation has shown that even after ¼ hour at 100° the antigen is incapable of giving rise to antibodies. In other words this antigen, on heating to 100° C. is rapidly converted into a haptene. Heating to 100° for 1 hour destroys the *in vitro* activities of this antigen as well. These properties remind one of those possessed by the flagellar antigen of *B. typhosus* as

described by Craigie (1931). For comparison, precipitin tests were carried out with a pure flagellar serum for *S. aertrycke*, prepared by absorbing an H + O serum with a non-motile aertrycke variant, thus leaving only H precipitins in the serum. The precipitating antigens were prepared by heating a suspension of agar-grown cultures of aertrycke (two slopes 2 days at 37° into 1 c.c. saline) at 60° for 1½ hours and centrifuging. The supernatant was subjected to 100° for varying periods in order to test the heat-resisting powers of its H component. The results of this experiment are shown in Table III:

TABLE III.

	Antigen dilutions.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.
H + O ærtrycke serum absorbed with an O variant of *S. aertrycke*	Non-motile ærtrycke antigen.	−	−	−	−	−	−	−
	Motile ærtrycke antigen .	++	++	++	++	++	++	+
	Ditto heated 1 hour 100° C. .	++	++	++	++	++	++	+
	Ditto heated 2 hours 100° C. .	−	−	−	tr.	+	tr.	−
	Ditto heated 3 hours 100° C. .	−	−	−	−	−	−	−

Pure flagellar serum resulting from absorption is unable to precipitate an O antigen, but can react with an H antigen even after it has been steamed 1 hour. After this amount of heating, H antigen, as is well known, is largely incapable of producing H antibodies in an inoculated animal; it has been haptenized. Heating for 2–3 hours destroys the *in vitro* reactivity of the flagellar substance as well. The flagellar antigen of *S. aertrycke* has behaved in these respects similarly to the envelope substance of *B. pestis*, but is more heat-stable.

The heat-resisting powers of the somatic antigen of *B. pestis* resemble those of the same antigen in *S. aertrycke* (compare Table III with Table IV). Both, unlike flagellar and envelope substance, are active *in vitro* after steaming for 3 hours. Moreover, there is not the ready haptenization at this temperature, for steamed cultures can in both cases be used for the production of antibodies. Plague vaccine steamed for an hour still occasions ready somatic antibody production in the rabbit.

TABLE IV.

	Antigen dilutions.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.
Aertrycke serum	Non-motile aertrycke antigen .	++	++	++	++	+	−
	Ditto heated 3 hours 100° C. .	++	++	++	++	+	−

AGGLUTININ REACTIONS OF B. PESTIS.

The agglutination of *B. pestis* has always been accompanied by difficulties. Sera of high titre are difficult to obtain. Cultures grown at 26° are found to be serum-sensitive, and to agglutinate to a considerable degree with normal rabbit sera; they are also unstable in salt concentrations; in fact they behave in the manner we associate with rough strains. Cultures grown at 37° are no longer serum or salt-sensitive but they are difficult to agglutinate, and with weak sera are apt to prove altogether inagglutinable, as Pulgher (1922) has already observed; it is obvious that the development of the gelatinous envelope has altered the agglutinating properties of the culture very considerably.

Quite different results are also obtained according to whether one uses living, 60° killed, or steamed suspensions for the agglutination test; the different extent to which virulent and non-virulent cultures produce envelope substance also influences results to some degree. The various modifications of the test may be summarized as in Table V:

TABLE V.—B. pestis *Agglutination.*

Culture.	Suspension.	Anti-envelope serum.	Anti-somatic serum.	Higher salt concentrations.	Normal serum.
Grown at 37° C.	Living	+	−	−	−
	Heated 60° C.	+	−	−	−
	Steamed	−	+	−	−
Grown at 26° C.	Living	+	+	+	+
	Heated 60° C.	+	−	−	−
	Steamed	−	+	+	−

It is clear that the agglutination of *B. pestis* depends on the interaction of the same antibodies and antigens as bring about the precipitation reaction. Anti-envelope serum will cause agglutination when it acts upon envelope-coated bacilli, but less well when the envelope is in solution after heating to 60°, and not at all when it is destroyed at 100°.

Anti-somatic serum has no action upon envelope-coated bacilli, but only upon those whose envelopes have been dispelled by steaming.

It is more difficult to obtain a high-titred envelope agglutination than it is to obtain a somatic one.

Cultures grown at 26° show a serum- and salt-sensitivity which is removed by heating to 60° or by steaming, in which latter case, however, the salt-sensitivity remains.

Suspensions heated to 60° are generally unsuitable, for while the heating damages envelope-agglutination, it apparently does not render the bacillus sufficiently accessible to the somatic antibodies for interaction to occur.

It is remarkable that a 37°-grown culture is still salt-stable after steaming, although the heating has destroyed the serological activity of the envelope substance and, one might have imagined, its stabilizing effect as well.

CONCLUSIONS.

1. The plague bacillus possesses two antigens, one contained in the gelatinous envelope and the other in the somatic portion of the bacterium.
2. The development of envelope antigen is furthered by growth at higher temperatures such as 37° C.
3. Envelope antigen is heat-labile, being haptenized after so short a period as ¼ hour at 100° C. and destroyed after 1 hour at that temperature.
4. Somatic antigen is more heat-stable; after 1 hour at 100° C. it is still capable of provoking antibody formation in the rabbit, and it still acts *in vitro* after 3 hours at 100° C.

REFERENCES.

CRAIGIE, J.—(1931) *J. Immunol.*, **21**, 417.
PULGHER, F.—(1922) *Arch. Schiff. Tropenhyg.*, **26**, 165.
ROWLAND, S.—(1914) *J. Hyg.*, Plague Supplement, III, 418.

STUDIES IN *B. PESTIS* ANTIGENS: II. THE ANTIGENIC RELATIONSHIP OF *B. PESTIS* AND *B. PSEUDOTUBERCULOSIS RODENTIUM*.

HARRY SCHÜTZE.

From the Bacteriological Department, Lister Institute, London.

Received for publication May 7th, 1932.

In view of the antigenic analysis of *B. pestis* described in a foregoing paper, it was of interest to see what the detailed serological relationship of this bacillus to *B. pseudotuberculosis rodentium* might be.

There have been conflicting accounts by various workers of the cross-agglutination and cross-precipitation reactions of the two organisms (Zlatogorov, 1904; MacConkey, 1908; Albrecht, 1910; Saisawa, 1913; Lerche, 1927; Arkwright, 1927; Zlatogorov and Mogilevskaia, 1928). Before proceeding to a comparison of the antigens of *B. pestis* and *B. pseudotuberculosis rodentium*, it will perhaps be well to recall a description of those possessed by the latter.

This organism is endowed with motility (a property rediscovered by Arkwright, 1927), and possesses both flagellar and somatic antigens. Schütze (1928) was able to show that the 18 strains examined by him fell into three groups, differentiable by their somatic antigen, whereas all possessed identical flagellar antigen. All but one of the strains belonged to Types I and II and were isolated from birds and mammals; the single strain forming Type III was isolated from man. Since then the author has diagnosed as also belonging to this Type III a non-motile organism isolated by Krishnamurti in India as the causal agent of bovine lymphangitis, and more recently, a representative of a new Type IV, differing in the same way as the other types do from one another, namely, in its somatic antigen, the flagellar antigen being identical with that of all strains in the species; this new type organism was isolated by Miss M. Rhodes at the Lister Institute from a marmoset dying in private possession in London.

Agglutination and absorption tests, as described in the author's previous paper (1928), establish the identity of the flagellar antigen throughout the species and the diverseness of the smooth somatic antigen in each serological type, these two being the only antigens concerned in an agglutination test when the cultures employed are smooth.

In the precipitin test, as will be shown below, three antigens apparently play a part. This test is carried out by adding two capillary drops of antigen to 5 capillary drops of a 1/5 dilution of anti-serum, incubating at 37° for 1 hour, and reading the result after standing in the cold room overnight. The precipitating antigen consisted of the supernatant of two slopes of a 3-day culture (grown at room temperature to favour the development of flagella), which had been suspended in 1 c.c. saline and heated to 60° for 1½ hours.

This third antigen doubtless represents the basal rough framework of the bacillus which, according to the theory of Bruce White (1929), is laid bare and comes into action in an agglutination test only when the organism loses its smooth specific somatic factor and becomes rough, but, when the organism is smooth, it is entirely covered by the specific antigen, and thus precluded from participation in the agglutination reaction. In the precipitin test conditions are different; here, the whole bacillus being lysed, the three antigens are in solution and ready to react with their respective antibodies.

In Table I this third antigen is demonstrated by cross-precipitation between a somatic pseudotuberculosis serum of Type I and antigens of Types II, III and IV. The antigen is seen to be common to the species, and absorption with Types II, III or IV will remove the corresponding antibody while leaving the type specific antibody intact.

TABLE I.—*Somatic Cross-Precipitation Between Pseudotuberculosis Types.*

Anti-somatic serum.	Antigen.	Result.
Ps. Type I unabsorbed. . .	Ps. Type I . . .	+
	Ps. Types II, III or IV .	+
Ps. Type I absorbed with Ps. Types II, III or IV	Ps. Type I . . .	+
	Ps. Types II, III or IV .	—

In Table II absorption of a serum containing somatic and flagellar antibodies with steamed suspension of the homologous type is seen to leave the flagellar antibody intact, this antibody reacting with the flagellar antigens of all four groups alike.

TABLE II.—*Flagellar Cross-Precipitation Between Pseudotuberculosis Types.*

Anti-flagellar + anti-somatic serum.	Antigen.	Result.
Ps. Type I unabsorbed . . .	Ps. Type I . . .	+
	Ps. Types II, III or IV .	+
Ps. Type I absorbed with Ps. Type I (steamed)	Ps. Type I . . .	+
	Ps. Types II, III or IV .	+

Thus in the precipitin test there is involved not only a heat-labile, flagellar antigen common to the species, and a heat-stable, somatic antigen specific to each type, but also a heat-stable antigen which is common throughout the four types.

Similar cross-precipitations and absorptions which include the antigens and sera of *B. pestis* demonstrate the fact that this third antigen is also possessed by the plague bacillus. In Table III one sees that a somatic pseudotuberculosis serum precipitates a plague somatic antigen, though not a plague envelope antigen, and that plague bacilli will absorb the type non-specific antibody from it just as did the heterologous pseudotuberculosis strains in Table I, but the type specific antibody is left intact.

TABLE III.—*Cross-Precipitation Between Somatic Pseudotuberculosis Serum and Plague.*

Pseudotuberculosis anti-somatic serum.	Antigen.	Result.
Ps. Type I	Ps. Type I	+
	Ps. Types II, III or IV	+
	Plague (somatic)	+
	Plague (envelope)	−
Ps. Type I absorbed with plague (somatic)	Ps. Type I	+
	Ps. Types II, III or IV	−
	Plague (somatic)	−

That there is no cross-precipitation between pseudotuberculosis flagellar antibody and *B. pestis* is seen in Table IV, where, after absorption with steamed pseudotuberculosis culture, flagellar antibody is left intact but no reaction with plague antigen occurs.

TABLE IV.—*Cross-Precipitation Between Flagellar Pseudotuberculosis Serum and Plague.*

Anti-flagellar + anti-somatic serum.	Antigen.	Result.
Type I absorbed with Type I (steamed)	Types I, II, III, or IV	+
	Plague (somatic or envelope)	−

Conversely in Table V, it is shown that while plague flagellar serum does not precipitate pseudotuberculosis antigen, plague somatic serum does, and that absorption with pseudotuberculosis culture removes all precipitin for *B. pestis* itself.

TABLE V.—*Cross-Precipitation Between Plague Serum and Pseudotuberculosis Types.*

Serum.	Antigen.	Result.
Plague (somatic)	Plague (somatic)	+
	Ps. Types I, II, III or IV	+
Plague (somatic), absorbed with Ps. Types I, II, III or IV	Plague (somatic)	−
	Ps. Types I, II, III or IV	−
Plague (envelope)	Plague (envelope)	+
	Ps. Types I, II, III or IV	−

It has been demonstrated, therefore, that the only somatic antigen possessed by *B. pestis* is also possessed by *B. pseudotuberculosis rodentium*, being, in fact, the heat-stable antigen which is common to all the pseudotuberculosis types, and which, it has already been suggested, represents the rough somatic antigen. This cross-precipitation of *B. pestis* and *B. pseudotuberculosis* establishes an interspecies relationship which is due to the possession of a common, rough, somatic antigen, and is the basis of the cosmopolitan agglutination of the author (1921); similar "rough" relationships in the coli-dysentery-salmonella groups have been investigated by Bruce White (1929). It would follow, then, that *B. pestis* is to be regarded as a bacterium which exists, as a rule, in the rough state, for it possesses, apart from the envelope antigen, merely this one rough somatic antigen.

The annexed diagram (Fig. 1) illustrates the antigens possessed by *B. pestis* and *B. pseudotuberculosis rodentium* and the extent to which they overlap. *B. pestis* develops at higher temperatures an envelope antigen which surrounds a basal rough antigen. This rough antigen is basal also for *B. pseudotuberculosis rodentium*, but, in this case, there exists a smooth, somatic antigen to which there is apparently no counterpart in *B. pestis* and, as well, in motile forms there develops flagellar antigen.

The agglutination reactions of *B. pestis* have always suggested that the organism is normally in a rough condition, *i. e.* possesses no smooth, somatic substance; deprived by growth at lower temperatures of its envelope which has a stabilizing effect, suspensions are found to be unstable in salt-solutions as low as 0·3 per cent. and to be sensitive to normal rabbit sera. Bessonowa and Lenskaja (1931) have published similar views, and report the occasional isolation of strains which, because of their salt-stability, they hold to be, on the contrary, smooth. It remains to be seen whether these so-called smooth

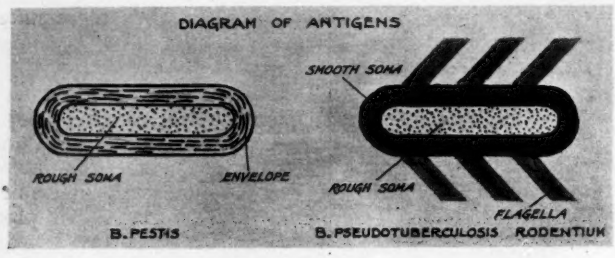


FIG. 1.

strains possess a species-specific somatic antigen, or owe their stability in salt-solutions to some other quality, *e. g.* an abnormal development of envelope substance.

CONCLUSIONS.

1. *B. pestis* possesses but two antigens, one corresponding to the envelope and one to the somatic substance.

2. *B. pseudotuberculosis rodentium* possesses three antigens: (i) a flagellar antigen which is common to all four types in the species; (ii) a somatic antigen which is type-specific and can be regarded as the smooth, somatic antigen; and (iii) a somatic antigen which, like the flagellar one, is common to all types; this, it is suggested, is the rough somatic antigen.

3. The antigen which *B. pestis* and *B. pseudotuberculosis rodentium* have in common is this rough somatic antigen; it is in virtue of it that serological relationship between the two species exists.

4. *B. pestis* is to be considered as, normally, existing only in a rough state, there being no smooth species-specific antigen present.

REFERENCES.

ALBRECHT, H.—(1910) *Wien. klin. Wschr.*, **23**, 991.
ARKWRIGHT, J. A.—(1927) *Lancet*, **1**, 13.

BESSONOWA, A., AND LENSKAJA, G.—(1931) *Rev. Microbiol.*, Saratov, **10**, 236.
LERCHE.—(1927) *Zbl. Bakt.*, Abt. I, Orig., **104**, 493.
MACCONKEY, A. T.—(1908) *J. Hyg.*, Camb., **8**, 335.
SAISAWA, K.—(1913) *Z. Hyg. InfektKr.*, **73**, 353, 401.
SCHÜTZE, H. L.—(1921) *J. Hyg.*, Camb., **20**, 330.—(1928) *Arch. Hyg.*, Berl., **100**, 181.
WHITE, P. B.—(1929) *J. Path. Bact.*, **32**, 87.
ZLATOGOROV, S. I.—(1904) *Zbl. Bakt.*, **37**, 345, 513, 654.
Idem AND MOGILEVSKAIA, B. I.—(1928) *Ann. Inst. Pasteur*, **42**, 1165.

STUDIES IN *B. PESTIS* ANTIGENS: III. THE PROPHYLACTIC VALUE OF THE ENVELOPE AND SOMATIC ANTIGENS OF *B. PESTIS*.

HARRY SCHÜTZE.

From the Bacteriological Department, Lister Institute, London.

Received for publication May 7th, 1932.

IT has been shown in a foregoing paper that the plague bacillus contains two antigens, one associated with the envelope and the other with the body of the bacterium, the former more readily produced at 37° C. than at 26° C. The prophylactic value of these two substances remained to be tested. Vaccines, accordingly, were prepared from a virulent strain; they were agar-grown at 26° C. and 37° C. for 3 days, the saline suspensions being standardized by counting and phenolized to 0·5 per cent.; sterilization took place at room temperature.

Rats were immunized by inoculating subcutaneously with 187 m., followed a week later by 374 m., a dose which corresponds to an eighth of that prescribed for human beings. Two weeks later a test dose of living *B. pestis* representing a thousand or more M.L.D. was given subcutaneously to the immunized animals and to uninoculated controls. The united results of several experiments are shown in Table I.

TABLE I.—*Comparison of Vaccines Agar-grown at 26° and 37° C.*

Number of rats.	Vaccine.	10 days after the test dose.
143	26° grown	36 per cent. survive.
192	37° ,,	72 ,, ,,
65 uninoculated controls		All dead in 4–5 days.

Growth at 37° has produced a vaccine that undoubtedly possesses greater prophylactic power than one grown at 26°. A practical disadvantage, however, of the higher temperature is the scanty growth that occurs and would hamper vaccine production on a large scale. While a Roux bottle will yield 200–300 thousand million organisms at 26°, only 20–30 thousand million can be expected at 37°.

For this reason, attention was turned to the possibility of improving the prophylactic value of the Haffkine broth vaccine by incubating the organism

at 37° rather than at 25°–30°, as is customary in Bombay, for, although this vaccine is of a more toxic nature, it possesses the merit of very simple manufacture.

Flasks of the acid digest broth, as prepared in Bombay, and of hormone broth, as recommended by Huntoon (1918) for massive growth of bacteria, were similarly seeded with *B. pestis*, and incubated for 6 weeks at both 26° and 37° C. The period of incubation was arbitrarily adopted from the usual process of manufacture of Haffkine's prophylactic, and it is possible that 6 weeks at 37° may not be the optimum period, but precipitin tests carried out at weekly intervals with the broth cultures as antigen, showed that envelope substance increased in amount with each additional week of incubation. These tests were, of course, performed with the centrifuged supernatants of the vaccines, and give no account of the envelope substance contained in the bodies of the microbes themselves. Sterilization took place at 56° for ½ hour.

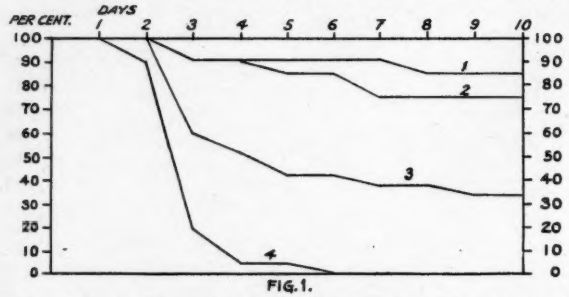


FIG. 1.

FIG. 1.—Rats (21 in each group) immunized with ⅛ c.c. vaccine. Percentage of survivors on consecutive days. 1 = 37° acid digest broth vaccine. 2 = 37° hormone broth vaccine. 3 = 26° acid digest broth vaccine. 4 = uninoculated controls.

In the first experiment recorded in Figs. 1 and 2, the immunizing effect of acid digest and hormone broths grown at 37° is compared with that of acid digest broth grown at 26°. The animals recorded in Fig. 1 were inoculated with ⅛ c.c., followed by ¼ c.c. a week later (*i. e.* together an eighth of a human dose); those in Fig. 2 were inoculated with half these amounts. A fortnight after the second inoculation a test dose, consisting of 3½ mgm. of spleen from an infected rat, emulsified in saline, and representing some 600,000 living plague germs, was injected subcutaneously. The graphs record the daily survival rate of the immunized batches, alongside that of a group of uninoculated controls.

In Figs. 3 and 4 a similar experiment is recorded which was carried out with a fresh set of vaccines; in this case ordinary tryptic digest broth and Haffkine's acid digest broth were employed. The test dose, given fourteen days after the second inoculation, consisted of ½ c.c. of a 2-day 37° broth culture inoculated with heart-blood from a plague-infected rat and contained 37 million living plague germs. The graphs again record the survival rate of the immunized and control animals. In both experiments the number of rats in each group was 21, except in the case of the 37° broth batches in Fig. 4, where the numbers were 40 and 42 respectively.

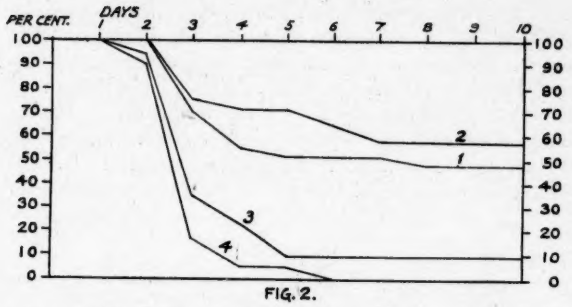


FIG. 2.

FIG. 2.—Rats (21 in each group) immunized with $\frac{3}{16}$ c.c. vaccine. Percentage of survivors on consecutive days. 1 = 37° acid digest broth vaccine. 2 = 37° hormone broth vaccine. 3 = 26° acid digest broth vaccine. 4 = uninoculated controls.

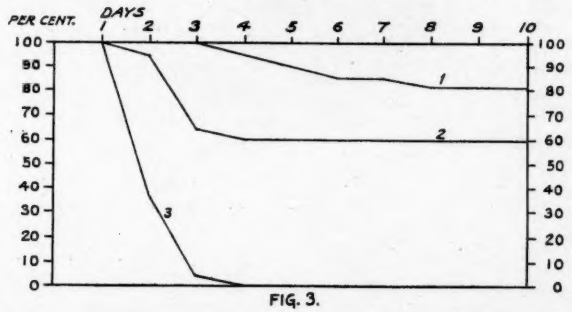


FIG. 3.

FIG. 3.—Rats (21 in each group) immunized with $\frac{3}{8}$ c.c. vaccine. Percentage of survivors on consecutive days. 1 = 37° acid digest broth vaccine. 2 = 26° acid digest broth vaccine. 3 = uninoculated controls.

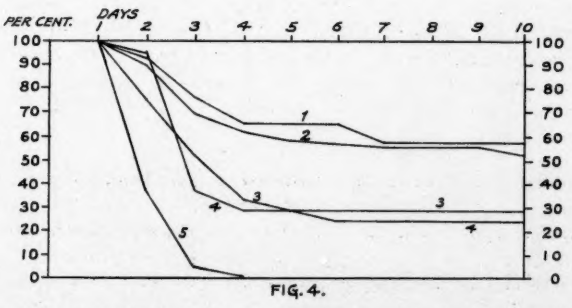


FIG. 4.

FIG. 4.—Rats immunized with $\frac{3}{16}$ c.c. vaccine. Percentage of survivors on consecutive days. 1 = 37° tryptic digest broth vaccine (40 rats). 2 = 37° acid digest broth vaccine (42 rats). 3 = 26° acid digest broth vaccine (21 rats). 4 = 26° tryptic digest broth vaccine (21 rats). 5 = uninoculated controls (21 rats).

It is plain that in the case of broth-grown vaccines, as in that of agar-grown ones, incubation at 37° has improved quite considerably their prophylactic value, and it seems likely that this is due to the greater extent to which envelope substance is produced at the higher temperature. That this increased potency is not accompanied by a corresponding exacerbation of toxicity was shown by tests carried out on rats and reported in Table II. Indeed every indication is that 37°-grown broth vaccine is less toxic, which is hardly surprising in view of the fact that cultures at this temperature are much less massive than they are at 26°.

Animals closely approximating in size were chosen for this experiment. Samples of the broth vaccines which had been kept without addition of phenol after sterilization by heating to 56° were used. The action of plague toxin is a rapid one; any deaths that occur take place within 24 hours. It was noted that bucks are considerably more resistant than are does, and the experiment was then confined to the latter.

TABLE II.—*Proportion of Deaths in Toxicity Tests on Rats.*

Vaccine.	Grown at—	Amount per 100-gm. rat injected intraperitoneally. ¾ c.c. Bucks.	¾ c.c. Does.	⅜ c.c. Does.	⅜ c.c. Does.
Acid digest broth (1)	26°	3 of 12	5 of 6	..	1 of 5
	37°	0 ,, 12	4 ,, 6	..	0 ,, 6
Tryptic digest broth (1)	26°	..	5 ,, 6	4 ,, 6	..
	37°	..	5 ,, 6	1 ,, 6	..
Acid digest broth (2)	26°	..	..	5 ,, 6	..
	37°	..	..	3 ,, 6	..
Tryptic digest broth (2)	26°	..	..	5 ,, 6	4 of 6
	37°	..	..	5 ,, 6	3 ,, 6

It has been shown in a foregoing paper that the two antigens existent in the plague bacillus, the envelope and the somatic substance, possess different degrees of resistance to heat. Envelope substance is completely haptenized by ¼ hour at 100°, while somatic substance resists that temperature for at least 1 hour. It would, therefore, be of interest to measure the heat-resisting powers of plague vaccine from the prophylactic point of view. In the following summary of an experiment will be seen the effect of steaming an agar-grown vaccine for one hour.

SUMMARY.

21 rats immunized with 37° agar-grown vaccine; 76 per cent. survive 10 days.

21 rats immunized with the same vaccine steamed for 1 hour, all dead in 5 days.

21 uninoculated control rats, all dead in 4 days.

The vaccine is thus rendered worthless, the rats inoculated with it resisting infection no more than do the uninoculated controls.

As half an hour at 100° haptenizes envelope but not somatic substance, it

must be concluded that envelope substance is the important ingredient of the vaccine.

In view of the opinion expressed in a foregoing paper that the somatic antigen of *B. pestis* is a rough one, this conclusion might be expected, for rough antigen is generally regarded as of little prophylactic value. Still, given in sufficiently big doses, even the somatic antigen seems capable of affording protection against a large infective dose. Naidu and Jung (1929) demonstrated that Lustig and Galeotti's (1897) nucleoproteid extract given in doses of 0·9 c.c. of a 0·2 per cent. solution, which probably represents one whole slope of culture, protected rats as well as their own Haffkine broth vaccine. Yet Lustig's extract contains, it would seem, only somatic and no envelope antigen. The author is indebted to Prof. Lustig, of Florence, and Prof. Pepeu, of Milan, for a sample of 0·2 gm. which was derived from 100 agar slopes. High concentrations of this extract in 1 per cent. sodium carbonate gave no precipitin

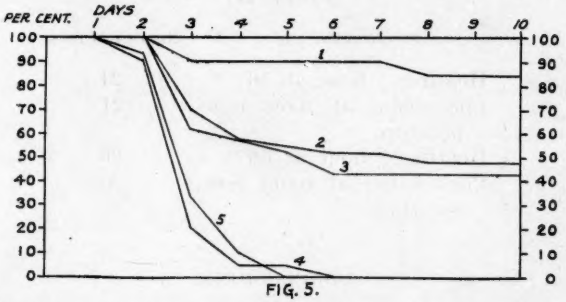


FIG. 5.

FIG. 5.—Rats inoculated with vaccine. Percentage of survivors on consecutive days. 1 = 21 rats with $\frac{1}{8}$ c.c. 37° acid digest broth vaccine. 2 = 21 rats with $\frac{3}{16}$ c.c. 37° acid digest broth vaccine. 3 = 21 rats with $\frac{1}{8}$ c.c. supernatant of 37° acid digest broth vaccine. 4 = 21 rats with $\frac{1}{8}$ c.c. supernatant of 37° acid digest broth vaccine, $\frac{1}{4}$ hour 100°. 5 = 21 uninoculated control rats.

reaction with an envelope serum, while with a somatic serum they gave an abundant precipitate.

In Fig. 5 is demonstrated the extent to which the effective antigens of a 37° broth-grown vaccine are in solution and free of the plague bacilli still maintaining in the vaccine. The vaccine having been centrifuged, the supernatant was used as such, and also after steaming for a $\frac{1}{4}$ hour. The curve representing the survivors after immunization with $\frac{1}{8}$ human dose of supernatant is plotted alongside one representing those after immunization with $\frac{1}{16}$ human dose of whole vaccine, and the experiment indicates that the supernatant is about half as effective as the complete vaccine. Steaming for $\frac{1}{4}$ hour, it will be seen, renders the supernatant useless as an immunizing agent.

The haptenization of envelope substance is very rapid at 100°, and the following experiment indicates that the process begins at much lower temperatures.

A 37° agar-grown vaccine was prepared by washing down 3-day cultures with 0·5 per cent. phenolized saline, half being sterilized by standing at room temperature and half by heating to 60° for 1 hour. The two vaccines were

then tested prophylactically in the usual way, 19 rats immunized with the unheated vaccine giving a survival rate of 53 per cent., 20 rats immunized with the 60° heated vaccine giving a survival rate of only 35 per cent. It would seem as if 1 hour at 60°, it must be added in the presence of 0·5 per cent. phenol, had appreciably damaged the prophylactic value of the vaccines.

It was for this reason taken into consideration whether even the usual sterilization at 56° for ½ hour (not in the presence of phenol) might not be damaging to the vaccine. Accordingly broth-growth cultures at the end of their 6 weeks' incubation at 37° were divided, half being sterilized at 56° for ½ hour before the addition of ½ per cent. phenol and half being sterilized by adding ½ per cent. phenol and standing at room temperature. From Table III it will be seen that heating to 56° has not lowered the prophylactic values of the vaccines.

TABLE III.

Vaccine.	Sterilization.	Number of rats.	Percentage survivors 10 days after test dose.
Acid digest broth culture	Heating ½ hour at 56°	21	57
	Phenolizing at room temperature	21	48
Tryptic digest broth culture	Heating ½ hour at 56°	20	65
	Phenolizing at room temperature	20	50

CONCLUSIONS.

1. Both agar- and broth-grown vaccines have their prophylactic potency increased by 50 to 100 per cent. by incubating the cultures at 37° rather than at 26°. The nature of the broth employed, whether acid digest, tryptic digest or hormone, does not affect the result.

2. As growth at 37° favours the development of envelope substance, this suggests that it is envelope antigen which is prophylactically important.

3. Heating to 100° for ¼ hour renders a plague vaccine useless. As this amount of heating haptenizes envelope substance while even after 1 hour at 100° somatic substance is capable of evoking antibody formation in the rabbit, it is again indicated that envelope antigen is prophylactically the important one.

4. Heating for 1 hour at 60° in the presence of 0·5 per cent. phenol is damaging to plague vaccine, but ½ hour at 56° before the addition of phenol does not lower the value of the vaccine as compared with sterilization by phenolization at room temperature.

5. Broth vaccines grown at 37°, though more potent, are less toxic than those grown at 26°.

REFERENCES.

HUNTOON, F. M.—(1918) *J. Infect. Dis.*, **23**, 169.
LUSTIG, A., AND GALEOTTI, G.—(1897) *Deut. med. Wschr.*, **23**, 227, 289.
NAIDU, B. P. B., AND JUNG, J. S.—(1929) *Ind. J. Med. Res.*, **17**, 199.